Water-use efficiency in perennial ryegrass (*Lolium perenne* L.)

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Plant Science

at Massey University, Manawatu,

New Zealand

Lekamalage Visna Yahani Weerarathne

2021

Abstract

Knowledge of genetic and physiological bases of drought responses and stress tolerance properties of pasture plants is an integral part of designing efficient pasture improvement programs to combat the consequences of climate change. However, experimental evidence or theoretical analyses on that aspect is sparse in the literature. Therefore, considering wider applications, high economic importance, and acknowledged poor tolerance of (*Lolium perenne* L.; PRG) to drought, the main aim of this research was to evaluate morpho-physiological trait responses that are linked to water-use efficiency (WUE) in different PRG populations from different sources in simulated summer drought cycles under a controlled environment. This study also included a quantitative genetic analysis conducted on the key traits to ascertain which traits are under genetic control for future breeding purposes.

Experiment 1 screened single potted PRG genotypes from three commercial cultivars with industry reputation for persistence for natural differences in morpho-physiological traits related to water use (WU) under simulated drought. Large within-population variation was observed for the measured traits which included, among others, WUE (g WU/g plant dry matter); shoot dry weight, SDW; leaf osmotic potential, OP; leaf relative water content; predawn leaf water potential; root dry weight at 20–50 cm depth, RDW_D; gravimetric soil moisture at 30–40 cm depth, SMC_D; post-cutting regrowth. Principal component analysis (PCA) identified important trait associations contributing to high WUE (i.e. WUE-OP-RDW_D trait association) and one related to higher SDW together with 'SMC_D conservation' indicative of 'true WUE' was used to make a divergent selection of 20 high- and 15 low-WUE genotypes (HWUE and LWUE, respectively). Experiments 2 and 3 were conducted simultaneously using the same methodology as Experiment 1 and inter-randomised in the same glasshouse space. Experiment 2 retested clones of HWUE and LWUE plants selected in Experiment 1 for consistency of trait expression across the two consecutive growing seasons, and also collected data for additional traits. Results confirmed that the key trait associations identified in Experiment 1 were almost identically expressed in Experiment 2. From data on additional traits, it was established that the accumulation of high molecular weight sugars in the shoots significantly contribute to 'true WUE' of a subset of PRG genotypes but, with the less involvement of gas exchange data under the conditions tested. It is speculated that enhanced mesophyll conductance of CO_2 might underlie this important trait association. However, the large majority of genotypes exhibited a 'SMC_D-depleting' trait association of WUE with improved gas exchange and maximum quantum efficiency of PSII, demonstrating late A.R. Blum's theory of WUE. Thus, selection of PRG for drought tolerance should consider yield and soil moisture data together to establish the most appropriate category of WUE trait association in improved cultivars. Experiment 3 investigated drought response trait associations in two further populations: a group of elite plants from a commercial plant breeder's

breeding program (CBL) and a PRG germplasm line derived from crossing Mediterranean and Middle Eastern seed accessions (MMEL) compared with those from the HWUE selection. PCA results showed that the major trait associations found in the yield and water relations data of elite subsets of CBL closely followed those of the HWUE selection, but elite MMEL plants exhibited typical summer dormancy characteristics where the average SDW of MMEL was 40% of lower than that of the CBL plants. Results further suggested that the company field evaluation system could benefit from the consideration of water relations traits, including WUE and associated traits like OP, as externally-measured selection traits for PRG drought tolerance. Experiment 4 evaluated quantitative genetic parameters of the key traits using the same methodology from Experiment 1 in a breeding population of 36 'half-sib (HS) families' under both stressed and non-stressed conditions. There were significant estimates of among- and within-HS family genetic variances, narrow-sense heritability, and predicted genetic gain estimates for the key traits, indicating high genetic potential of each trait for breeding purposes under the conditions tested. However, the correlated response to selection of each trait pair comprising highly genetically correlated morpho-physiological traits with WUE was lower than that gain from the single-trait selection, accommodating further research questions on the efficacy of indirect and multi-trait selection of key traits. Based on the current results, it was found that the direct selection of PRG for WUE or concurrent selection for OP, RDW_D, SMC_D, and SDW or RGS traits under drought is advisable. Furthermore, significant quantitative genetic parameters estimated for WUE under non-stressed conditions together with high genetic correlation observed for WUE between stressed and non-stressed conditions suggested that a PRG population can potentially be selected for this trait at early growth stages or before imposition of water deficit.

Key words: Drought, genetic gain, heritability, osmotic potential, perennial ryegrass, deep root dry weight, shoot dry weight, deep soil moisture, water-use efficiency.

Dedication

For plant physiologists, agronomists, plant breeders, academia, and farmers

Acknowledgements

I would like to take this opportunity to place on record my hearty thanks to all those who have immensely contributed to my PhD research work and thesis in numerous ways.

First of all, I must mention my deepest sense of appreciation for my chief supervisor Prof. Cory Matthew for his valuable guidance, kindness, patience, excellent understanding, and the endless support given for my work through all the good and bad times and also for the great amount of time spent on my thesis corrections amidst his busy schedule. I am also very much thankful for all the external opportunities that Prof. Cory created for me to widen my horizons in academic and professional fields and also, in various extra-curricular events during my stay in New Zealand. Secondly, my first co-supervisor Prof. Ignacio Lopez also deserves special thanks for his valuable advice of all sorts, encouraging words, and emotional support given throughout my work even in his challenging times. I would also like to express my appreciation and special thanks to my second co-supervisor Dr. Zulfi Jahufer from Agresearch for his valuable input in Chapter 6 of my thesis and his professionalism and vast knowledge in plant quantitative genetics that helped me to expand my knowledge in another dimension of agriculture. Apart from the above-mentioned support, all three supervisors were very friendly to me so that I could talk freely with them about almost everything including my personal matters and I'm genuinely grateful for that.

A special note of thanks goes to Massey University and The T.R. Ellett Agricultural Research Trust for funding me to follow my full-time PhD degree at Massey University and also to New Zealand Grassland Association, New Zealand Society of Plant Biologists, Massey Internal Scholarships Committee, and the German Academic Exchange Service for supporting me with timely and casual funds including travel grants to take part in national and international conferences, academic events, and collaborative research in a different institution during my PhD tenure.

I would like to express my appreciation and gratitude for the invaluable support given by Mark Osborne (especially for his very friendly and timely assistance),

Lesley Taylor (was my all-time helper), Zoe Yang (also a great friend), and Vladimir Kaul (Plant Growth Unit, Massey University) and Kay Sinclair (the best ever helper), Chris Rawlingson, Brian Smith, Sunmeet Bhatia (Ag/Hort, School of Agriculture, Massey University) in many ways whenever and wherever it was necessary. I highly appreciate the technical support given by four summer intern students from Massey University (especially Jacinta Harrop and Alisha Harrop), nine (three helpers in each experiment: Mr. Dong Wencheng, Ms. Nie Minghe, and Ms. Wang Yan for Experiment 1 (2017); Ms. Wang Xiaofang, Ms. Yu Shuang, and Mr. Mingyu Li for Experiment 2 and 3 (2018); Mr. Sha Baiping, Ms. Wang Wenxiao, and Ms. Zhang Yi for Experiment 4 (2019)) visiting Masters students from Ningxia University (China), and three student interns from University of Angiers (France), respectively. I am also thankful to professional visitors at Massey University; Prof. Lilian Pereira, Prof. Zhang Yongmei, Dr. Hu Haiying, and Dr. Wang Zhanyi for their priceless support given in different challenges during my PhD research work. Without them all, I wouldn't have been able to complete the massive workload that I had from the point of setting up this series of experiments to the bulk data collection and post-experiment analyses in each experiment, this successfully. I am also grateful for the support given by former PhD students Lulu He and Hossein Ghanizadeh with their scientific ideas related to my research.

I would like to express my sincere gratitude to Alan Stewart and Tom Lyons from PGG Wrightson Limited, New Zealand, for giving me an opportunity to work with two groups of the company's PRG breeding germplasm lines with necessary logistic and timely support and for helping us to get our PRG selection to the next level using their resources.

Even though the outcome of the supplementary analysis of Experiment 4 of my PhD research that I conducted in the Grassland Chair, School of Life Sciences, Technical University of Munich, Germany, is not presented in this thesis, that particular work greatly helped my professional and personal growth during my PhD tenure. For that reason, I would like to heartily thank Prof. Hans Schnyder, Dr. Rudi Schäufele, and all the staff members and postgraduate students (especially Regina Hirl and Jianjun Zhu) of the Grassland Chair for their great support, academic guidance, invaluable learning experience, and warm welcome offered to me during my stay in Germany.

I’m thankful for the short-term research grant of the German Academic Exchange Service (DAAD) for the financial support provided.

I would also like to express my sincere and heartfelt thanks to my undergraduate supervisors Prof. Lalith Suriyagoda (especially for his statistical support and invaluable academic guidance and directions given for my continuous personal growth) and Prof. Buddhi Marambe (who is my all-time academic inspiration) of the Department of Crop Science, Faculty of Agriculture, University of Peradeniya, for being pillars of my academic strength that I developed during my tenure as a Lecturer since 2015 and the academic success that I’ve attained in this PhD degree. I would also like to thank Prof. Palitha Weerakkody (my most treasured mentor) for his valuable guidance and emotional support given during hard times. I’m also grateful to University of Peradeniya for granting me an official study leave to pursue my PhD studies.

I would like to extend my warmest appreciation and gratitude to my boyfriend Dr. Maulik Mungalapara Patel especially for his great understanding during hard times and endless emotional support given whenever it was needed. During a 6-month period when I was stranded in Freising, Germany (also during the unexpected stay in Sri Lanka for the last few months before completion) due to Covid-19 restrictions, he was always there for me to absorb all my emotional breakdowns and to provide healing, encouragement, and reinforcement for my thesis writing in return. For last but not least, my younger sister, beloved parents, and younger brother and also some special friends deserve another special token of appreciation for showering me with love, care, and attention and for being there for me throughout this 4-year long PhD journey.

Contents

Glossary of Abbreviations

Abbreviation	Full name/ meaning	Units
ABA	Abscisic acid	
AFS	Among-family selection	
AL1	Actinic light is turned on to drive photosynthesis	µmol/m^2/s
AL2	Actinic light is turned off and far-red light is turn-on	µmol/m^2/s
ANOVA	Analysis of variance	
ASW	Argentine stem weevil	
ATP	Adenosine triphosphate	
AWFS	Among-and within-family selection	
BLUP	Best Linear Unbiased Predictor	
CBL	Commercial breeding line	
CF	Chlorophyll fluorescence	
Ci	Intercellular CO_2 concentration	µmol/m^2/s
Ci/Ca	The ratio between intercellular CO_2 concentration and atmospheric CO_2 concentration	
CR	Correlated response of traits	
DW or DM	Leaf dry weight or dry matter	
ET	Plant evapotranspiration rate	mmol/m^2/ s
ETR	Electron transport rate	
EUW	Effective use of water	g/g
F'_m	Maximum fluorescence level of light-adapted leaves	
F'_o	Zero level fluorescence 'in the light'	
FC	Field capacity	
F_o	Zero fluorescence level of dark-adapted leaves	
F^o_m	Maximum fluorescence level of dark-adapted leaves	
F_t	The steady-state value of fluorescence immediately prior to the flash at the light-adapted state of the leaf	
F_v	The level of fluorescence immediately before the second saturating flash (variable fluorescence)	
FW	Leaf fresh weight	g
G×E	Genotype-by-environment interaction	
GCA	General combining ability	
GLM	Generalized Linear Model	
HI	Harvest index	
HMWWSC	High molecular weight WSC	mg/g
HS or HSF	Half-sib or half-sib family	
HWUE	Low water-use selection	

LMR	Leaf mass ratio	%
LMWWSC	Low molecular weight WSC	mg/g
LWP	Predawn leaf water potential	MPa
LWUE	High water-use selection	
MANOVA	Multivariate analysis of variance	
MB	Measuring light beam	
MC	Mesophyll conductance	
MDA	Malondialdehyde	
MMEL	a seed line derived from crossing Mediterranean and Middle Eastern accessions	
MS	Mass of soil	g
MW	Mass of water	g
NIWA	National Institute of Water and Atmospheric Research	
NPQ or qN	Non-photochemical quenching	
NZ	New Zealand	
OA	Osmotic adjustment	
OP	Leaf osmotic potential	MPa
p	Statistical probability	
PCA	Principal component analysis	
PDW	Total plant dry weight	g/plant
Pn	Photosynthesis rate	µmol/m^2/s
PRG	Perennial ryegrass (*Lolium perenne* L.)	
PS	Photo system	
PWP	Permanent wilting point	
qP	Photochemical quenching	
QTL	Quantitative trait loci	
r	Pearson correlation	
rA	Genetic correlation	
RAWHC	Readily-available water holding capacity	
RDW_D	Root dry weight at 20–50 cm depth	g/plant
RDW_T	Root dry weight at 0–20 cm depth	g/plant
RDW_{Tot}	Total root dry weight	g/plant
REML	Restricted Maximum Likelihood procedure	
RGS	Post-cutting regrowth score	0–5 score
RHL	Leaf relative humidity	%
RSR	Root to shoot ratio	%
RWC	Leaf relative water content	%
SC	Stomatal conductance	mmol/m^2/s
SCA	Specific combining ability	
SDW	Shoot dry weight	g/plant
SEM	Standard error of mean	
SMC_D	Gravimetric soil moisture content at 20–50 cm depth	MW/MS, %
SMC_T	Gravimetric soil moisture content at 0–20 cm depth	MW/MS, %

SP	Saturating flash of light	
SPAC	Soil-plant-atmosphere continuum	
SWU	Plant soil water use	g Water/pot
TE	Transpiration efficiency	
T_L	Leaf temperature	^oC
TP	Turgor potential	MPa
TW	Leaf turgid weight	g
VPD_L	Leaf water vapor pressure deficit	KPa
WFS	Within family selection	
WHC	Water holding capacity	
WP	Water potential	MPa
WSC	Water soluble carbohydrates or sugars	mg/g
WU	Pot water use	g Water/pot
WUE	Agronomic water-use efficiency (g WU/g DM)	g/g
WUE_{AE}	Instantaneous water-use efficiency (Pn/ ET)	
WUEi	Intrinsic water-use efficiency	
$\Delta^{13}C$	Carbon isotope discrimination	
$\Delta^{18}O$	Oxygen isotope discrimination	
ΔG_c	Predicted genetic gain	
σ^2_b	Replicate effect	
σ^2_c	Column effect	
σ^2_{fb}	HSF× replicate effect	
σ^2_{fc}	HSF× column effect	
σ^2_{fr}	HSF× row effect	
σ^2_f	Row effect	
φPSII	The efficiency of Photosystem II	
h^2_n	Narrow-sense heritability	
σ^2_n	Genetic variance	
σ^2_A	Additive genetic variance	
σ^2_f	Among-HSF effect	
$\sigma^2_{f/s}$	Within-HSF effect	
σ^2_ε	Error variance	

List of figures

water-use (LWUE) groups across four different soil moisture stages imposed in Experiment 2.

Figure 4.6 Post-cutting regrowth score (RGS; 0–5) of three test cultivars (Nui, Samson, and Trojan) of low water-use (HWUE) and high water-use (LWUE) groups as recorded when watering was completely withdrawn at the end of M3 (45–55% field capacity) and plants were defoliated leaving 5 cm of stubble to support regrowth, drawing on residual soil moisture and scored after 7 days in Experiment 2.

Figure 4.7 (A1) Root dry weight for 0–20 cm soil depth (RDW_T), **(A2)** Root dry weight for 20–50 cm soil depth (RDW_T) and **(B1)** gravimetric soil moisture content for 10–20 cm soil depth (SMC_T), **(B2)** gravimetric soil moisture content for 40–50 cm soil depth (SMC_D) across three different (M2, M3, and M4) soil moisture stages as recorded for 'Nui', 'Samson', and 'Trojan' cultivars of low water use (HWUE) and high water use (LWUE) selections in Experiment 2.

Figure 4.8 Change in predawn leaf water potential (LWP), leaf osmotic potential (OP), and leaf relative water content (RWC) of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across the three different soil moisture deficit stages imposed in Experiment 2.

Figure 4.9 Patterns of change in concentrations of (A) high molecular weight water soluble carbohydrates (HMWWSC), (B) low molecular weight water soluble carbohydrates (LMWWSC), and (C) proline of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across three different soil moisture deficit stages imposed in Experiment 2.

Figure 4.10 Change in net photosynthesis (Pn) and stomatal conductance (SC) of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across three different soil moisture deficit stages imposed in Experiment 2.

Figure 4.11 The key chlorophyll fluorescence measurements representing the photochemistry and the overall photosynthetic capacity of the three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups under intense drought (M3) in Experiment 2.

Figure 7.1 Identifying key traits of a model perennial ryegrass plant for high WUE based on the ecophysiological signature of water deficit response in the current results.

List of Tables

Table 5.2 Eigenvalues and principal component (PC) co-efficients and p-values in the ANOVA output on PC scores (including ANOVA mean scores for each PC of trait data from each germplasm tested) of the first five of nine PCs in PCA 5.3, compiled using stacked trait data of nine morphological and water relations traits in 38 commercial breeding line (CBL) genotypes, 20 low water-use selection (HWUE) genotypes from Experiment 1, and 14 Mediterranean cross (MMEL) genotypes as measured at M2.

Table 5.3 Principal component (PC) eigenvalues and coefficients for the first five PCs of PCA5.4 that includes nine yield and water relations traits and two commercial company field test scores (MFS and MPNS, see below) of 38 perennial ryegrass genotypes of the commercial breeding line (CBL) compiled for the investigation of consonance between glasshouse and field testing.

Table 6.1 Overview of data: Half-sib family (HSF) population means (Pop mean) with range and standard error of trait means (±SEM) and their statistical probabilities (p) for replicates (Rep), HSFs, genotypes-within-HSF population (Gen), and genotypes-within-HSF (Gen [HSF]) effects of measured traits at each consecutive measurement phase (MS; M1 and M2) and 'p' of MS and interaction effects between MS and HSF and MS and Gen for the measurements repeated at both M1 and M2.

Table 6.2 Principal component (PC) coefficients for the first five and three PCs generated by PCA6.1 and PCA6.2 of 17 and 9 morpho-physiological and water relations data, respectively, as obtained from 180 genotype means of two clonal replicates of 360 test plants (excluding check plants) of the HS family population, measured at M2.

Table 6.3 Narrow-sense heritability, significant variance components with their standard errors (±); genotype-within-HS families and among-HS families and residual error and coefficient of variation of measured plant traits as estimated among the 36 HS families at M2.

Table 6.4 Genetic correlation coefficients between heritable plant trait pairs ($p<0.05$) and predicted genetic gain (ΔG_c) of each trait by single trait selection at

30% of selection pressure in the tested perennial ryegrass HSF plant population under imposed drought.

Table 6.5 Correlated response to selection (CR%) estimates of heritable traits when perennial ryegrass genotypes are simultaneously selected for two traits (primary and secondary) within the tested HSF population at 30% of selection intensity under imposed drought.

Table 6.6 Estimates of genetic correlation coefficients, narrow-sense heritability, and predicted genetic gain of selected traits (i.e. that were recorded as heritable traits at M1), if they were selected from the tested HS family population under an assumed 30% selection pressure in both M1 and M2.

List of Appendices

Table 4.A4 Pearson correlation co-efficients estimated for the trait data of selected 35 perennial ryegrass genotypes between Experiment 1 and 2 that were measured at M2 (55−65% FC) in both experiments.

Table 5.A1 Untransformed trait means and their standard deviation (SD) values of 38 genotypes of the commercial breeding line (CBL), a two-criterion selection of the best four CBL plants considering field score (as the prioritized feature) and glasshouse WUE (CBL-elite) namely, P19, P17, P21, and P37, the best four CBL plants considering high glasshouse WUE together with the highest possible field-score (CBL-HWUE) namely, P19, P17, P32, and P9, 14 plants of the Mediterranean cross (MMEL), and 20 genotypes of the high water-use efficiency selection (HWUE) from Experiment 1 and 2 as measured at M2 (55–65% FC).

Figure 6.A1 The row (R)-column (C) experimental design incorporating two clonal replicates of 36 perennial ryegrass HS families from a breeding population as test plants, and four clonal replicates of two HS families as check plants (CH) to statistically test for spatial effects on plant growth.

Table 6.A2 Cross-correlations between the PC scores for the first five PCs of PCA6.1 and PCA6.2 performed for the fifteen and nine water relations traits, respectively, of the HS family population measured at M2.

Table 6.A2 Pearson correlation co–efficients of measured traits of 180 genotype means of two clonal replicates of 360 test plants (excluding check plants) of the HS family population, measured at M2

Chapter 1

Introduction

1.1 Background

New Zealand's economy is underpinned by pastoral farming, and New Zealand's livestock industry, currently includes 26.8 million sheep, 3.9 million beef cattle, and 6.3 million dairy cows (StatNZ, 2019). The contribution of the dairy industry is particularly notable. New Zealand's dairy produce generates annual earnings that are two and a half times greater than the meat sector, more than three times the forestry sector, and ten times the wine sector (NZIER, 2019). Dairy produce earned around $NZ18.1 billion in 2019, making up around 28% of the total value of New Zealand's merchandise exports. By volume, New Zealand is the largest milk exporter globally and produces more than 30% of internationally traded dairy produce. However, the competitive advantage of New Zealand dairy exports in the international dairy market mainly depends on the low-cost pastoral farming systems (He, 2016). Dairy farmers commonly re-sow about 10% of their pastures annually with ryegrass-dominant seed mixes, to maintain high productivity (Erickson, 2017).

Lower rainfall totals compared to the long-term average of the pasture growth requirement during summer and autumn are becoming increasingly common in many northern and eastern areas of New Zealand. Consequent soil moisture deficits have caused a noticeable decline in the pasture productivity and in turn affected livestock farming returns, with a severe summer-autumn shortage of feed for livestock. In particular, such weather events have often resulted in a significant drop in lambing ewe and beef cattle numbers in recent years as farmers will often lower stock numbers to mitigate perceived risks associated with climate-related inter-annual variation in feed supply (Hutching & Moor, 2017; Johnston, 2013; NZGovernment, 2019; StatNZ, 2019; Tait, 2016).

Perennial ryegrass (*Lolium perenne* L.; PRG), with desirable features of high palatability and digestibility, easy establishment and management, and comparatively good persistence, has been the most widely grown grass species in New Zealand farming systems (Cyriac et al., 2018; Frame, 1989; Hannaway et al., 1999; Lee et al., 2012; Moot et al., 2009; Wilkins, 1991; Williams et al., 2007).

However, reduced summer production of PRG due to its acknowledged poor tolerance of drought has caused feed shortages for livestock. As a consequence, on dairy farms production cost related to supply of supplements is increased, reducing profits (Macdonald et al., 2011). Moreover, the National Institute of Water and Atmospheric Research (NIWA) has recently predicted that severe summer moisture deficits will be more frequent in the future than they have been historically, thus imposing a significant additional cost on New Zealand's livestock farming (Hutching & Moor, 2017). Hence, there is strong industry interest in improving summer drought tolerance of PRG.

Genetic improvement programs in livestock and pastoral industries have been central to the development of the New Zealand's agricultural sector since the 1930s (Lee et al., 2012). Given the importance of pasture drought tolerance because of New Zealand's heavy economic dependence on pastoral industries, germplasm screening for persistence has recently received attention in New Zealand PRG breeding programs (Cyriac et al., 2018; Lee et al., 2012). Although improved summer performance has been addressed indirectly by the use of Spanish germplasm in pasture improvement programs in New Zealand since the 1990s (Stewart, 2006), the possibility of selecting PRG directly for traits related to maintenance of productivity under water deficit has received little attention in New Zealand plant breeding. Water-use efficiency (WUE) is one such productivity trait that may contribute to both productivity and survival of plants under drought (de Almeida Silva et al., 2012; Ferguson et al., 2018). However, drought tolerance is a complex plant characteristic that is governed by a combination of traits including morphological, physiological, and anatomical traits (Blum, 2005; Blum, 2009; Blum, 2011). Hence, careful selection of morpho-physiological trait combinations to optimize plant water economy, together with knowledge of trait heritabilities, is essential to achieve high gains in WUE when considering drought tolerance traits.

1.2 Objectives

Water-use efficiency of forage grasses is historically a little-researched topic, but in view of emerging climate change issues, this trait is becoming important for improving forage productivity. As PRG is New Zealand's primary forage grass species, the main objective of this thesis was to ascertain key water relations traits

that could be used to screen PRG populations for elite genotypes exhibiting improved WUE and summer performance. It was hypothesised that physical traits such as soil exploration by roots and regrowth rate together with leaf physiological traits are diversely associated with the WUE of PRG populations under simulated summer drought conditions. Considering very little information exists on genetic aspects of water relations traits of PRG, this research also aimed at evaluating quantitative genetic parameters including the heritability of WUE and associated traits to inform pasture breeders which traits can feasibly be changed in breeding PRG cultivars for drought tolerance through mass selection.

1.3 Thesis structure

Excluding this introduction (Chapter 1), there are six interconnected chapters in this thesis (Figure 1.1).

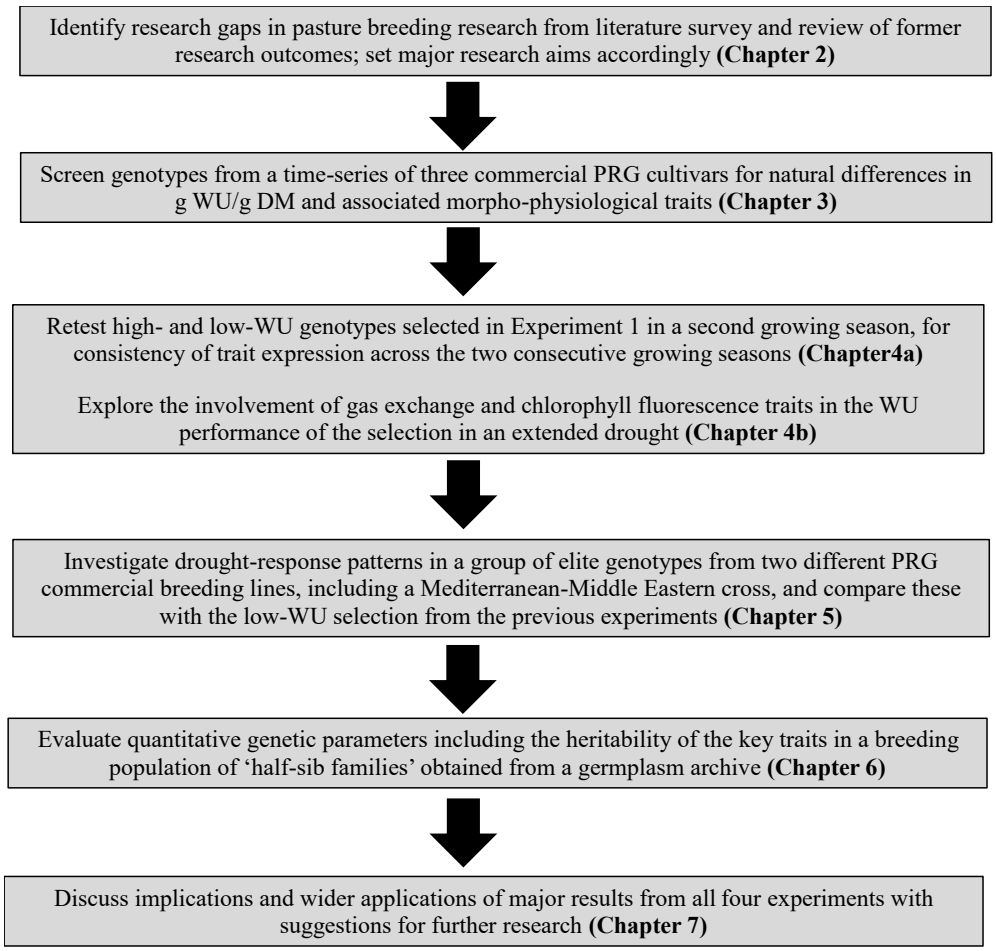

Figure 1.1 A flow diagram indicating the sequence of experiments conducted during this study and the link between chapters of the thesis (Abbreviations: PRG, Perennial ryegrass; WU, Water use; DM, Shoot dry matter).

Chapter 2 provides a literature review in which topics covered include: the origin, distribution, adaptation, and biology of PRG, the importance of *Epichloë* endophyte symbiont in New Zealand-grown PRG, drought-related terminologies, drought impacts on pasture production in New Zealand, plant adaptations to drought stress, plant traits involved in the key drought-response patterns, physiological breeding strategies for plant drought tolerance, breeding and selection approaches for PRG drought tolerance, PRG breeding techniques, history and recent trends in PRG breeding in New Zealand, major quantitative genetic parameters, and implications of quantitative genetic parameters in breeding PRG for high WUE and drought tolerance. Four chapters (i.e. Chapters 3, 4, 5, and 6) each report separate glasshouse experiments conducted from 2017 to 2019, providing background, objectives, and methodology and then discussing the results and drawing conclusions.

The present PhD research is informed by the results and future research suggestions of previous Massey University PhD studies. Hussain (2013) reported a wide variation in WUE between genotypes of the cultivar Grasslands Samson and proposed selection for this trait as a promising direction. He (2016) showed that osmotic adjustment (OA) is an important contributing trait to PRG performance during summer moisture deficit. Based on these results, visiting Brazilian researchers Carnivalli and Garcia (Garcia, 2015) conducted a 'proof of concept' study with 81 genotypes of three PRG cultivars. This study provided 'methodology experience' for simulating field drought conditions in potted single PRG genotypes in a glasshouse, and also identified traits and trait associations of interest for further study in investigation of the ecophysiology of water deficit tolerance in PRG (Garcia, 2015) (see also 1A.1).

Accordingly, Experiment 1 (Chapter 3) was conducted from September 2017 to March 2018 and screened a larger total number of genotypes (n=440) from a time-series of three commercial PRG cultivars for natural differences in WU per gram of forage dry matter production and associated water relations traits. Experiments 2 and 3 (Chapters 4 and 5, respectively) were conducted simultaneously using the same methodology and inter-randomised in the same glasshouse, from August 2018 to February 2019. Experiment 2 retested, in a second growing season, results of high and low WU genotypes selected in Experiment 1, for consistency of performance across the two consecutive growing seasons. Experiment 3 investigated drought

response patterns in two different PRG breeding lines in comparison to the low-WU selection: a group of elite genotypes from a commercial pasture breeder's breeding program and a PRG germplasm line of Mediterranean origin, a naturally more arid environment. Experiment 4 (Chapter 6) was conducted from October 2018 to April 2019 and again used methodology from Experiment 1 to perform water relations trait measurements similar to those in Experiments 1–3. The point of difference in Experiment 4 was that the tested plants were a breeding population of 'half-sib families' from a germplasm archive to evaluate the quantitative genetic parameters including the heritability of the key traits. The final chapter (Chapter 7), summarises the main findings, discusses the implications of the results for PRG breeding, and makes suggestions for future research.

Chapter 2

Literature review

2.1 Perennial ryegrass

2.1.1 Origin, distribution and, adaptation

Perennial ryegrass (*Lolium perenne* L.; PRG) (NRCS, 2006) occurs naturally in Europe, temperate Asia and, North Africa (Cunningham et al., 1994; Hannaway et al., 1999; Lamp et al., 1990; Thorogood, 2003; Thorogood & Hayward, 1991). Perennial ryegrass freely interbreeds with Italian ryegrass (*Lolium multiflorum* Lam.) and PRG varieties bred from these two species are now utilized for forage and turf purposes throughout the temperate world (Bothe et al., 2018; McDonagh et al., 2016; Sampoux et al., 2011; Sampoux et al., 2013), most extensively in the United States, Europe, Japan, Australia and, New Zealand (Cunningham et al., 1994; Thorogood, 2003; Thorogood & Hayward, 1991). In New Zealand, it is the most widely used temperate forage grass species in intensive pastoral production systems (Charlton & Stewart, 1999; Cyriac et al., 2018; Harmer et al., 2016; Lee et al., 2012; Stewart, 2006).

Among the temperate forage grass species, PRG is palatable, highly nutritious and digestible to livestock. Also, this species is comparatively easy to establish and maintain, able to withstand treading and hard grazing, adapts easily to a wide range of edaphic situations, and usually forms a compatible mixture with white clover and a wide range of other pasture grass and legume species (Frame, 1989; Hannaway et al., 1999; Wilkins, 1991; Williams et al., 2007). Thus, its usage exceeds six million hectares in Australasia, primarily as PRG-based pastures (Moot et al., 2009). Perennial ryegrass is best adapted to cool, moist climates and grows best on fertile, well-drained soils despite having a good tolerance to a wide range of soil conditions, including low soil pH, and waterlogging among others (Cunningham et al., 1994; Thorogood, 2003). However, PRG is intolerant of drought and high temperatures, particularly when day time temperatures exceed 31°C and night time temperatures exceed 25°C (Sullivan & Sprague, 1949; Thorogood, 2003) and soil moisture deficit (Attia et al., 2015; Cyriac et al., 2018; Rogers et al., 2019; Thorogood, 2003;

Tuberosa, 2012; Ullaha et al., 2019). Hence, PRG growth and development can be significantly negatively impacted by periodic summer drought conditions.

2.1.2 Biology of PRG

2.1.2.1 Cytogenetics

Perennial ryegrass is naturally diploid (2n=14) (Cooper, 1951). Colchicine-induced autotetraploids have been developed for forage purposes that are typically larger plants with bigger cells, higher tissue water content, and larger seeds (Thorogood, 2003).

In New Zealand, a large component of commercial PRG breeding activity centres around cultivars derived from *L. perenne* and *L. multiflorum* (Brock, 1983; Burgess & Easton, 1986; Easton & Fletcher, 2007; Humphreys et al., 2010; Lee et al., 2012; Rumball, 1970). Hybridisation with meadow fescue (*Festuca pratensis*) and back-crossing to PRG has also been used to develop commercial *L. perenne* cultivars, with the objective of harnessing drought tolerance traits from the meadow fescue parentage (Easton & Fletcher, 2007; Easton et al., 1994; Lee et al., 2012; Stewart, 2006) and also to introduce the *E. uncinata* endophyte from meadow fescue (Barker et al., 2015; Wiewióra et al., 2015).

Perennial ryegrass also exhibits a partial fertility with some species of the genus *Festuca*. As a result, natural hybrids between *L. perenne* and the Bovinae section of the tribe Festuceae (i.e. *F. arundinacea* or *Schedonorus arundinaceus* (Schreb.) Dumort (NRCS, 2006), tall fescue; *F. gigantean*, giant fescue) have been observed in the wild (Jenkin, 1955; Jenkin, 1933; Peto, 1933; Terrell, 1966). In addition, successful hybrids between *Lolium* species and members of the Ovinae section of the tribe Festuceae (i.e. *F. rubra*, red fescue) have been produced artificially (Jenkin, 1933; Terrell, 1966).

2.1.2.2 Modes of reproduction

The main mode of propagation in PRG is by seeds, while clonal ramets can also be used as the planting material (Thorogood, 2003).

Most temperate PRG have a dual induction requirement for floral initiation (i.e. the transition at the apical meristem from new leaf production to formation of spikelet primordia). The primary induction is facilitated by low temperature and short days during winter. The secondary induction requires a transition from the first induction with long days, which can also be enhanced by moderately high temperatures during late spring and early summer (Evans, 1960; Heide, 1994). Thus, vernalization (exposure to 6°C, generally for at least 6 weeks) followed by a long photoperiod (>14–16 hours) can effectively induce flowering of PRG (Heide, 1994). Perennial ryegrass vernalization is possible in both seeds and seedlings, although the rate of vernalization (when exposed to suitable conditions) is greater in seedlings than in seeds (Evans, 1960).

Perennial ryegrass is an allogamous and wind-pollinated species (Thorogood, 2003). In particular, PRG has a two-locus (S and Z) multi-allelic gametophytic incompatibility system (Cornish et al., 1980), which prevents self-pollination and subsequent inbreeding depression (Bean & Yok-Hwa, 1972; Cunningham et al., 1994; Jenkin, 1931). Theoretically, four different categories of pollination compatibility can be generated; fully compatible, three-quarters compatible, half-compatible and, fully incompatible, as a result of pair-wise ryegrass crosses (Jenkin, 1933). Nearly 32% of seed set can be achieved through obligate-selfing despite the fact that a naturally-developed self-incompatibility system exists in most PRG genotypes (Thorogood & Hayward, 1991). Therefore, PRG cultivar improvement is normally based on the development of advanced progenies through the exploitation of the high genetic diversity (i.e. selection of elite parent plants with high general combining ability) that exists in a given PRG population due to its outcrossing behaviour (Casler, 1995).

Human-manipulated inbreeding methods, that are typically used in cross-pollinated grain crop species, have seldom been employed for PRG breeding programs (Cornish et al., 1979; Thorogood, 2003). However, recent PRG research has made it possible to predict the compatibility level between parent plants using marker-assisted selection and such information may allow the exploitation of heterosis (resulting in ~83.33% of hybrid vigour) PRG within populations (Pembleton et al., 2015).

2.1.3 Perennial ryegrass in New Zealand

New Zealand's economy largely relies on the success of the pastoral industries (Lee et al., 2012; Morris, 2009). About 53% of the total land area of New Zealand is covered by grassland of various forms with about 74% of the grassland area (37% of the total land area) devoted to pastoral agriculture (Williams et al., 2007). The main farmed species by land area comprise 77% sheep, 11% dairy cows, 9% beef cattle, and 3% deer (Moot et al., 2009). The historic practice of mixed species sowings (Gould, 1974; Smallfield, 1970) was later replaced by one of relying mainly on PRG. Perennial ryegrass as the sole grass, sown with white clover (*Trifolium repens*) has now become the most common choice for new sowings over much of New Zealand (Charlton & Stewart, 1999; Cyriac et al., 2018; Goh & Bruce, 2005), especially on lowland and higher fertility pastures (Lee et al., 2012; Moot et al., 2009). Other pasture species commonly sown in New Zealand (with white clover) include Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) and together with PRG, these account for 98% of new pasture sowings on arable pasture land in the country (Pyke et al., 2004). Thus, PRG has become the most widely used temperate pasture grass species in New Zealand (Cyriac et al., 2018; Lee et al., 2012) despite the fact that it performs poorly during hot dry conditions and is prone to infection by crown and stem rust diseases (Charlton & Stewart, 1999; Lancashire & Latch, 1970).

2.1.4 Role of *Epichloë* endophyte in New Zealand PRG pastures

An additional feature of PRG use in the pastoral industry in New Zealand is that PRG seeds are normally sold infected with a known strain of *Epichloë* endophyte (Fletcher, 2012; He, 2016; Zhou, 2013). *Epichloë* endophyte is one of the most widely studied symbiotic fungal endophytes with respect to insect deterrence (He, 2016; Leuchtmann, 2003; Philipson & Christey, 1986) and persistence (Amalric et al., 1999; Hahn et al., 2008; He, 2016; Kane, 2011; Ravel et al., 1997; Thom et al., 2010) of cool season grasses including PRG.

Asexual *Epichloë* endophyte species, such as *E. festucae* var. *lolii* (formerly known as *Neotyphodium lolii*), *E. coenophiala* and, *E. uncinata* generally transmit from the host species or mother plant to seeds through vertical transmission (Christensen et

al., 2008; Philipson & Christey, 1986; Zhang et al., 2017). There are also sexual *Epichloë* species, such as *E. festucae*, *E. typhina*, and *E. amarillans* that are mostly associated with the 'choke disease' in grasses and disseminate through horizontal transmission with the aid of external sources (i.e. wind and insects) (Leuchtmann, 2003).

Endophytes were first introduced to New Zealand through PRG seed importations from Europe in the early 19th century and endophyte presence in PRG in New Zealand was first recorded by J.C. Neill in 1941 (Latch, 1966; Latch & Christensen, 1982). Even though laboratory-cultured endophyte had proven non-toxic (Cunningham, 1949; Cunningham, 1958), endophyte in plants in the field was linked to an animal health problem known as 'ryegrass staggers'. This was later found to be caused by the lolitrem group of endophyte alkaloids (Easton et al., 2001; Fletcher & Harvey, 1981; Fletcher et al., 1991; Gallagher et al., 1981). Similarly, tall fescue endophyte (*Epichloë coenophialum*) carried ergovaline alkaloid (i.e. a vasoconstrictor) that was recognised earlier as a mammalian toxin causing lameness in cattle (Bacon et al., 1977). Thus, tall fescue was sold 'endophyte-free' to overcome ergovaline toxicity problems in New Zealand for many years. For PRG, elimination of endophytes was initially explored as the solution to overcome the ryegrass staggers problem (Latch & Christensen, 1982). However, PRG without endophyte was found to be prone to insect attack and this attracted significant pasture research (di Menna et al., 1976; di Menna et al., 2012; DiMenna et al., 1992; Keogh et al., 1996).

The reduced performance of PRG without endophyte was eventually traced to greater insect attack. Then, an endophyte alkaloid, peramine, was identified as being involved in insect deterrence (Mortimer & Di Menna, 1983; Prestidge & Gallagher, 1988). As the importance of endophyte became evident, detrimental effects of endophytes on herbivorous pest insects were studied by many researchers (Gallagher et al., 1981; Mortimer & Di Menna, 1983; Prestidge et al., 1985), particularly on Argentine stem weevil (ASW) (Prestidge et al., 1985). An initial survey conducted in New Zealand, that attempted to find endophyte-infected PRG plants with low levels of lolitrem B, found endophyte strains producing low levels of lolitrem B and high levels of peramine from an international seed collection in the Margot Forde

Germplasm Centre, Palmerston North, New Zealand (Tapper & Latch, 1999). With that discovery, the crucial role of *E. festucae* var. *lolii* was redefined towards both pest tolerance and persistence and PRG improvement programs were reoriented accordingly since the early 1980s (Easton et al., 2001). As the next step, techniques for isolating endophyte strains from plants and re-inoculating them into elite pasture germplasm lines, with an ideal combination of endophytes began to be developed in 1985 (Latch & Christensen, 1985). After a few unsuccessful attempts (one of which, 'Endosafe' endophyte was found to sometimes produce ergovaline), a safe endophyte strain named AR1 which produces peramine, but not lolitrem B or ergovaline was released in 2000 and provided protection against ASW and pasture mealy bug (Thom et al., 2013). Thus, AR1 endophyte is currently licenced to be inoculated into many PRG cultivars by reputable New Zealand seed companies for exports off-shore (i.e. Australia and Chile) and overseas evaluations (i.e. the USA, Europe, and Argentina) (Johnson et al., 2013). Nevertheless, further research was warranted because AR1 endophyte did not provide a comprehensive protection against key pasture pests other than ASW and mealy bug (e.g. African black beetle and root aphid) with the peramine alkaloid (Popay & Baltus, 2001). In 2007, AgResearch introduced an endophyte strain named AR37 which did not produce peramine, ergovaline or lolitrem, but produced janthitrem alkaloids and conferred a broader insect deterrent ability than that of AR1 (Buckley & Warren, 2014; Hume et al., 2007; Pennell et al., 2005; Popay & Hume, 2011; Popay & Thom, 2009). AR37 endophyte was initially licenced to PGG Wrightson Ltd. and at first appeared only in their cultivars, but after two years it became available to other seed companies and is now available in a range of cultivars by other seed companies (Johnson et al., 2013). At this time, various other new endophyte strains were commercially developed, including Endo 5 by AgResearch and NEA2 by NZ Agriseeds Ltd., with low levels of ergovaline for the protection against black beetle (Popay & Hume, 2011).

Research evidence shows that subsequent inclusion of endophyte in PRG seed sold in New Zealand positively impacted on the agronomic performance (i.e. improved yield and tiller survival) of PRG cultivars (Popay et al., 1999; Prestidge, 1982; Rasmussen et al., 2007). A few studies have asserted that *Epichloë* endophyte promotes the regrowth of PRG plants by adjusting its metabolism in response to soil drought (Hesse et al., 2003, 2005; Marks & Clay, 2007; Ravel et al., 1997; Zhou,

2013). However, evidence confirming drought tolerance benefits of PRG endophyte has been elusive (He et al., 2017).

For research purposes, one of the major problems associated with the presence of known or unknown endophyte strains in test pasture cultivars is possible confounding effects of pasture-endophyte interactions on the cultivar performance (Kerr, 1987). Moreover, Casler (1995) stated that the genetic diversity of endophytes in foreign breeding materials (i.e. Spanish and North African germplasm), that have been introduced to the local PRG germplasm over the years (Stewart, 2006), may affect yield rankings of advanced breeding lines or newly introduced cultivars Easton et al. (2001). Hence, it is clear that research work on PRG performance should consider the endophyte strain status of the lines or varieties included in the experiment. In that case, there are two commonly used methods to check the endophyte status in grass tillers before setting a yield trial or a drought experiment: (1) microscopy examination (Clark et al., 1983; Latch & Christensen, 1982; Simpson et al., 2012) and (2) immuno-detection (Clark et al., 1983; Edwards et al., 2007; Groppe & Boller, 1997; Latch & Christensen, 1982). For experimental purposes, endophytes in growing PRG grass tillers and seeds can also be eliminated by treating them with fungicides (i.e. benomyl, dichlorobutrazol, triadimefon, etaconazole, propiconazole, prochloraz) (Latch & Christensen, 1982; Siegel et al., 1985) or by storing seeds at high temperatures and high humidity levels (Siegel et al., 1985).

2.2 Drought

2.2.1 Drought-related terminologies

Drought is one of the most damaging environmental stresses for plant growth and development (Abid et al., 2018; Attia et al., 2015; Chaves, 2002; de Almeida Silva et al., 2012; Jaleel et al., 2009) and the damage is aggravated by increase in magnitude and frequency of drought occurrences (Abid et al., 2018; Ludlow & Muchow, 1990; Wilhite & Glantz, 1985).

Drought impacts on plants may depend on a number of factors such as soil properties, plant characteristics, and environmental factors including temperature and humidity (Kramer, 1988; Kramer & Boyer, 1995; Ludlow & Muchow, 1990).

Consequences of a drought event are generally quantified in terms of the soil water content as a percentage of field capacity (FC) (Boutraa, 2010), the degree of plant dehydration (Kaiser, 1987), number of days of plant survival and production under drought (Huang et al., 1998a; Huang & Fry, 1998b), and water balance calculations (i.e. proportion of water used up of the total water input) (Hayatu & Mukhtar, 2010). Thus, soil moisture deficit is an important consequence of drought (i.e. except for high temperature) and one way to evaluate the level of drought on a regional scale by quantifying the difference between potential evapotranspiration and water input through rainfall or irrigation (Matthew et al., 2012). Plant production is generally compromised whenever soil available water supply is below the plant's water demand that is required to satisfy the rate of evapotranspiration (Kirkham, 2005; Kramer & Boyer, 1995).

Readily-available water (for plant uptake) is often taken to be 50% of soil water held within the soil moisture range between FC and permanent wilting point (PWP) (Kirkham, 2005; Kramer & Boyer, 1995). Field capacity is defined as the soil moisture content remaining after saturation by heavy rain or saturating irrigation followed by downward drainage under gravity, and typically occurs at a soil matric potential of about –0.03 MPa (Kramer & Boyer, 1995). Permanent wilting point (PWP) refers to the amount of water per unit weight of soil where water is held so tightly to the soil matrix that it cannot be absorbed by plant roots, causing plants to wilt beyond recovery. Permanent wilting point is often assumed to occur at a soil matric potential of –1.50 MPa, although some crop plants can withdraw water to a more negative soil matric potential than –1.50 MPa (Kirkham, 2005). The water volume held between FC and PWP is often referred to as the soil 'available water holding capacity' (AWHC), and indicates the volume of water that is theoretically available (but not all is readily available) in soil to support plant growth or survival during soil drought (Kirkham, 2005). Kramer & Boyer (1995) report the FC of a 'Hano sand' and 'Chino silty clay loam', respectively, as 45 and 489 g H_2O/g dry soil, and PWP of the same soils as 22 and 150 g H_2O/g dry soil, giving an AWHC of 23 and 359 g H_2O /g dry soil, respectively, for the two soils. However, plant growth and yield is likely to be negatively affected before PWP is reached, and in agricultural drought, plants generally have to draw on non-readily available water depending on prevailing weather conditions, biological characteristics of the specific

plant including the plant growth stage, and the physical and biological properties of soil (Mishra & Singh, 2010). This drawdown of non-readily available water by plant roots is most likely to happen even after 50% of the soil WHC is exhausted. Generally, fine-textured soils (e.g. sit loam, clay loam) hold more readily available water than coarse-textured soils (e.g. sand and sandy loam) (Kramer & Boyer, 1995).

Wilhite & Glantz (1985) identified four basic categories of drought: meteorological, agricultural, hydrological, and socioeconomic drought. Among these, agricultural drought is of the greatest importance to the farmer, and refers to a period with declining soil moisture and consequent impairment of crop yield and profitability (not necessarily crop failure). The definition of agricultural drought makes no reference to a threshold value of readily-available soil water that will substantially restrict crop growth and yield (Mishra & Singh, 2010; Wilhite & Glantz, 1985). This is because the onset of agricultural drought will vary between situations, depending on meteorological and hydrological conditions and also biological and phenological characteristics of the plants and as well as soil hydraulic properties (Hisdal et al., 2000; Mishra & Singh, 2010; Wilhite & Glantz, 1985).

2.2.2 Drought impacts on pasture production in New Zealand

Annual rainfall in most areas of New Zealand ranges from 600 to 1600 mm (Mackintosh, 2001), with slightly higher rainfall in winter than in summer months. Optimal production of PRG in warm summer temperatures requires 5–6 mm rainfall or irrigation per day to meet pasture transpiration demand. Thus, almost all farming regions of New Zealand experience soil moisture deficit in an average-rainfall year (Matthew et al., 2012; Moot et al., 2009). The typical seasonal pattern in most pastoral farming areas of New Zealand is the depletion of the soil water reserves in summer months until it is relieved by autumn rains (Lee et al., 2012; Matthew et al., 2012; Thomas, 1990). As a consequence, the growth and development of PRG is generally restricted in summer dry seasons (Bothe et al., 2018; Cyriac et al., 2018; Lee et al., 2012; McDonagh et al., 2016; Sampoux et al., 2011; Sampoux et al., 2013) resulting in moisture-driven seasonal fluctuations in pasture productivity (Cyriac et al., 2018; Lee et al., 2012).

In New Zealand pasture land, water supply to PRG roots is likely to fall below the PRG plant's water demand when soil moisture deficit exceeds 100 mm. Significant herbage yield losses are possible when moisture deficit exceeds 150 mm (Matthew et al., 2012; McAneney et al., 1982), although as noted above, these thresholds may vary with factors such as soil texture (Mishra & Singh, 2010). In particular, in the Northern and Eastern parts of the North Island and the Eastern regions of the South Island of New Zealand, soil moisture deficit typically develops between the months from September to April (Johnston, 2013; Mackintosh, 2001). According to Johnston (2013), some parts of the North Island have received 50–70% of the expected average rainfall levels in summer for the past few years. These recent drought events have caused a large loss to pasture productivity, the dairy industry, and the total economy of New Zealand. The impact of low rainfall and poor pasture growth, was estimated by DairyNZ to have reduced total milk production by 260 million litres, resulting in an economic loss of around $NZ 130 million over the period of 2007–2017 (Johnston, 2013). Similarly, dairy farm production losses due to medium-scale drought events that occurred in Northland in recent years were estimated to be around 23% of the potential production (Hutching & Moor, 2017).

Recent evaluation of the projected consequences of climate change indicates more frequent drought events are likely in some areas including Waikato, Wairarapa, and Marlborough in near future (Johnston, 2013). Thus, Northland, South Auckland, Waikato (including Coromandel, Hauraki and, Matamata-Piako), Hawke's Bay and, Bay of Plenty were officially declared as drought-prone areas in New Zealand by the Ministry of Environment in 2017 (Hutching & Moor, 2017). Climate predictions also emphasized that common pasture growing areas in New Zealand will experience 5–10% more drought occurrences by the year 2050 than in the recent past, and this change will hamper the productivity of pastoral and dairy industries at large (Johnston, 2013). Hence, improving PRG for drought tolerance may have positive impacts for dairy production during dry summers (Charlton & Stewart, 1999; Cunningham et al., 1994; Lee et al., 2012; Rogers et al., 2019; Thorogood, 2003; Yates et al., 2019).

2.2.3 Plant adaptations to drought stress

Levitt (1980) described three major strategies by which plants withstand drought; (i) drought escape, (ii) dehydration avoidance or dehydration postponement (i.e. maintenance of turgor and tissue volume through continuing water uptake, reduction of water loss, and change in cell wall elasticity) and, (iii) dehydration tolerance or protoplasmic tolerance. The 'drought escape' strategy refers to the ability of a plant to complete its life cycle before developing severe drought stress (Levitt, 1980), an example being the annual legume, subterranean clover (*Trifolium subterraneum*). By definition, drought escape is not a strategy of temperate perennial grass species including PRG, as the life cycle of these grasses extends beyond one growing season (Johnson & Asay, 1993).

Drought resistance of PRG implies dehydration avoidance and/or dehydration tolerance trait responses, and these should be given more attention when breeding PRG for drought resistance (Johnson & Asay, 1993). Plant survival due to dehydration avoidance usually occurs when a given genotype expresses its capability to remain hydrated as the soil drought develops (Blum & Tuberosa, 2018). With this drought response, plants are capable of maintaining key physiological mechanisms and net assimilation at low to moderate levels (Blum, 2005). The strategy of dehydration tolerance comes into play if a plant can delay tissue death and cell mortality up to a very low plant water status under severe drought, but this also depends on the resilience of the plant metabolism of a given genotype (Blum, 2005; Blum & Tuberosa, 2018). Thus, the concept of dehydration tolerance is mostly applicable for plants grown in naturally arid regions. Such plants may reconstitute their membranes and be fully functional within hours of rewatering, even after reaching a critical level of tissue dehydration at which plant cell membrane function has already been disordered (Gaff, 1980).

Unlike temperate grasses, grasses adapted to naturally dry conditions including some PRG originating from Mediterranean or North African regions can endure prolonged severe drought through a survival strategy called 'summer dormancy' (Volaire et al., 2009; Volaire et al., 1998). This strategy is identified by four main features: (1) a substantial reduction in leaf production and expansion, (2) senescence of mature foliage, (3) dehydration of surviving organs, and (4) formation of resting

organs as an optional strategy (Laude, 1953). There are two levels of inherent summer dormancy in drought-resistant grass species: (a) complete dormancy, which is characterised by the growth reduction that involves the dehydration and senescence of the whole canopy (including the growing point) for a greater degree of soil water conservation and (b) incomplete dormancy, characterised by the reduction in new leaf growth, moderate leaf senescence, and enhanced levels of dehydrin and fructan that may help maintaining cell membrane integrity under drought and post-drought (Laude, 1953; Nie & Norton, 2009).

Summer dormancy response of forage grasses including cocksfoot (Norton et al., 2006; Volaire et al., 2009) and PRG (Nie & Norton, 2009) has been well described as a major drought survival strategy. Also, plant traits associated with summer dormancy strategies have unintentionally been included in to the development of pasture cultivars for drought tolerance (Casler et al., 1996; Easton et al., 2011; Silsbury, 1961). However, it was considered an undesirable strategy for temperate pasture production systems (Matthew et al., 2012) because such plant drought responses tend to be triggered by summer temperatures even when there is sufficient supply of water for moderate growth (Volaire & Norton, 2006). To the contrary, New Zealand farmers would prefer to exploit the summer pasture growth potential that exists to minimise supplementary feed costs (Matthew et al., 2012).

Blum (2005) and Luo (2010) used the term ‘drought resistance’ to describe genotypes showing high yield under a severe drought challenge, and considered that such plants have the capability to resist adverse consequences of drought by adjusting plant physiological mechanisms. However, several authors have suggested that the term ‘drought tolerance’ is more appropriate than ‘drought resistance’ as plants are literally incapable of resisting drought and plants may endure drought impacts through adaptive traits that provide strategies to mitigate drought impacts (Barker & Caradus, 2001). For PRG, drought tolerance is an extremely complex trait, and there are many gaps in the research on this topic. Thus, comprehensive studies to discover key drought-response patterns of PRG are warranted.

2.2.4 Plant traits involved in the key drought-response patterns

Drought impairs plant growth and disturbs plant water relations through a number of complex physiological and biochemical mechanisms at cellular and whole-plant levels (Abid et al., 2018; Attia et al., 2015; Blum, 2005; Chaves, 2002; Ji et al., 2012; Jones, 1979; Manavalan et al., 2009; Maseda & Fernández, 2016; Sallam et al., 2019; Xoconostle-Cazares et al., 2010). At initial stages of a mild to moderate drought, plants restrict the rate of photosynthesis mainly through partial or complete stomatal closure (Baker, 2008; Chaves, 2002; Cornic, 2000; McCree & Richardson, 1987; Tezara et al., 1999). As the drought progresses, stomata remain closed and secondary drought adaptations involving the photosynthetic apparatus (e.g. non-photochemical quenching of chlorophyll fluorescence) are triggered. These plant drought responses protect photosynthetic reaction centres and to avoid possible membrane damage, disturbed activity of adenosine triphosphate (ATP) synthesis-mediated enzymes, and subsequent cell damage and cell death, depending on the duration and intensity of the drought event (Amalric et al., 1999; Baker, 2008; Banks, 2018; Björkman & Demmig, 1987; Cielniak et al., 2006; Filek, 2006; Krause & Weis, 1991; Li et al., 2006; Lu & Zhang, 1998; Paknejad et al., 2007). Plants tend to display a range of morpho-physiological trait adaptations to withstand drought most of which are directly or indirectly linked to stomatal behaviour of plants (Chaves & Oliveira, 2004; Chaves, 2002; Chaves et al., 2003). Blum (2005) asserted that evaluating actual plant WUE (g dry matter/g WU) may result in selection of plants which restrict transpiration water loss through stomatal control, likely resulting in reduced net assimilation and eventually low yield. Therefore, this author advocated identifying crop growth strategies for effective use of water (EUW), which hinge around traits ‘maximizing soil water capture while diverting the largest part of the available soil moisture towards stomatal transpiration’ to ensure that a crop does not run out of water before maturity. Thus, Blum (2009) considered that WUE is basically a function of plant’s EUW which is not a plant trait to be improved through selection (See further discussion in Section 2.2.4.1 below).

In addition to reduced water loss by increased diffusive resistance or partial stomatal closure, primary drought adaptations also include several morphological adaptations: (1) leaf rolling due to deflation of bulliform cells (Begg, 1980; Frank et

al., 1996; Turner, 1986a), (2) increased water uptake with prolific and deep rooting behaviour (Abid et al., 2018; Blum, 2005; Chaves, 2002; Lopes & Reynolds, 2010; Manavalan et al., 2009), (3) leaf area reduction as a collective result of slow leaf expansion, longer leaf appearance interval or reduced tiller appearance rates, and greater leaf senescence (traits that are undesirable for pasture productivity) (Blum, 2005; Turner, 1986a), (4) inclined leaves or modified leaf angle (Ludlow & Muchow, 1990), and (5) hairy, shiny, and succulent leaves to reduce transpiration loss (Barker & Caradus, 2001; Jones et al., 1980b; Leafe et al., 1980; Ludlow & Muchow, 1990) and OA.

OA refers to the active accumulation of solutes leading to decreased osmotic potential (OP) in response to declining leaf water potential, thereby maintaining high leaf relative water content and turgor in plant cells (Blum, 2017; Serraj & Sinclair, 2002). This drought response is of utmost importance to ensure plant physiological mechanisms or plant metabolism is uninterrupted during prolonged drought (Blum, 2017; Blum & Tuberosa, 2018; Cielniak et al., 2006; Manavalan et al., 2009; Serraj & Sinclair, 2002). Thus, the accumulation of low-molecular-weight osmolytes in plant cells, including glycine, betaine, proline, and other amino acids, organic acids, polyols, water soluble carbohydrates, and inorganic ions plays a vital role in sustaining plant cellular functions and metabolic activities under drought (Abid et al., 2018; Delauney & Verma, 1993; Gomes et al., 2010; Khalil et al., 2016; Samuel et al., 2000; Sánchez et al., 1998; Shahidi et al., 2017; Solařová et al., 2016; Verbruggen & Hermans, 2008).

Plant growth substances such as salicylic acid, auxins, gibberellins, cytokinins, and abscisic acid (ABA) are generated in shoots and roots in response to drought, and these also modulate beneficial drought adaptations in plants (Chaves, 2002; Easlon et al., 2014; Farooq et al., 2012; Quarrie & Jones, 1979; Yan et al., 2017; Zhang et al., 2015). For example, ABA synthesis in roots is an initial plant drought response that regulates stomatal movements under drought. Polyamines, citrulline, and several metabolic enzymes in plants act as antioxidants to reduce adverse effects of water deficit under drought (Chaves, 2002; Fu & Huang, 2001; Gill & Tuteja, 2010; Hameed et al., 2013; Jiang & Huang, 2001a; Zhang et al., 2015) by improving leaf water status and adjusting cell membrane properties (Farooq et al., 2009).

Another important drought response in some plants is the adjustment of plant phenology that avoids possible yield compensations by shortening the vegetative growth duration of plants. This strategy ensures that the most critical plant developmental stages happen prior to drought (i.e. early maturity and drought escape) (Abid et al., 2018; Blum, 2005; Chaves, 2002; Edwards et al., 2016; Ludlow & Muchow, 1990). However, for temperate perennial grass species, plant phenological adjustments are less important (except for seasonal adjustments noted for stomatal characteristics (Knapp, 1993)) as they are exposed to drought events that are typically relieved with the rains at the end of summer or beginning of autumn, with continual pasture growth for several years (Johnson & Asay, 1993).

Farooq et al. (2012) suggested that one of the strategies by which plant drought stress tolerance can be achieved is mass screening and breeding of plants for the target traits. In that case, success can be achieved in any plant species if the selection is focused on the right traits or beneficial plant drought responses (Abid et al., 2018; Arab et al., 2019; Attia et al., 2015; Blum, 2005; Chaves, 2002; Cooper et al., 2014; Manavalan et al., 2009).

2.2.4.1 Water-use efficiency

Scarcity of water in summer seasons is placing pressure on dairy farmers to utilize water more efficiently, and as result, WUE of forages is becoming an important criterion for sustainable dairy production (Neal et al., 2011; Rogers et al., 2017). The knowledge of actual plant WU, its sensitivity to water stress, and tactics of efficient WU have been extensively applied in scheduling irrigation for pasture species in Australasia and South Africa (Heermann et al., 1990; Rogers et al., 2017; Truter et al., 2016). Therefore, in this thesis, WUE will be calculated and presented as: g WU/g DM (i.e. greater WUE is indicated by a numerically smaller value of WUE).

Theoretically, WUE can be expressed either as instantaneous or intrinsic WUE (WUE_{AE} or WUEi; reflecting a physiologist's perspective), which refers to the amount of CO_2 assimilated per unit of water transpired (Condon et al., 2004; Flexas et al., 2016; Lopez et al., 2019; Medrano et al., 2002; Read et al., 1992) or as agronomic WUE (WUE; reflecting a farmer's perspective) (Blum, 2005; Blum, 2009; Johnson & Asay, 1993; Passioura & Angus, 2010; Ullaha et al., 2019).

Agronomic WUE is an integrated and long-term measure that refers to the ratio between the total shoot biomass production (root biomass is rarely measured) and the total amount of plant water consumption (Blum, 2009; Ludlow & Muchow, 1990; Passioura & Angus, 2010). In most drought experiments, WUEi is generally used when defining WUE of a given crop because conventional glasshouse experiments that are performed by pot-weighing studies to estimate WUE are considered to be laborious, time-consuming and are less representative of field conditions (Feldman et al., 2018; Moghaddam et al., 2013). In recent research where plant drought tolerance was inferred from improved WUEi, carbon ($\Delta^{13}C$) or oxygen ($\Delta^{18}O$) isotope discrimination was often adopted as the proxy and more easily measurable trait in most crop species (Adiredjo et al., 2014; Akhter et al., 2010; Condon, 2020; Ehdaie et al., 1991; Rebetzke et al., 2002) including grass species (Ebdon & Kopp, 2004; Ghannoum et al., 2002). However, it's still unclear to what extent these proxy measures truly capture the WUE signal in terms of plant WU per unit of dry matter produced, particularly in a pasture plant.

Despite the practical importance of the WUE trait, the whole WUE-concept has been largely disregarded when breeding crops considering either WUE (as a direct measure) or WUEi (as an indirect measure) for drought tolerance (Blum, 2005; Blum, 2009). Blum (2005) argued that easily measurable proxies of WUE (i.e. WUEi or $\Delta^{13}C$) would be likely to result in selection of plant genotypes for traits that aid stomatal closure and reduced transpiration at the leaf-level under drought. Blum (2005) further opined that reduced net assimilation due to stomatal control may result in reduced yield and thus, evaluating plant's effective use of water at the whole-plant level (g WU/g DM) would be more realistic than measuring WUEi to define drought tolerance in plants. However, selecting high WUE plant genotypes is primarily equivalent to reduced WU by its definition and is unlikely to be a plant trait to be improved though selection (Blum, 2009). Alternatively, he felt higher WUE may arise from low WU by plants of smaller size, because of a range of drought-response patterns triggered under moisture deficit. In advocating for a focus on EUW instead of WUE, Blum (2009) observed that OA would be a likely contributing trait, conferring improved water capture by roots, and ability to maintain transpiration at lower plant water status.

Contrary to the opinion of Blum and others that the WUE trait is not a useful selection criterion for plant drought tolerance, there are a few recently published examples where heritability of WUE of C3 species was positively linked to drought tolerance in *Arabidopsis thaliana* (Ferguson et al., 2018), apple root stocks (Lopez et al., 2017) and C4 *Setaria* grass species (Feldman et al., 2018), demonstrating its applicability for plant breeding purposes. Corroborating this finding, Massey University research of (Hussain, 2013) reported that some single-potted genotypes of PRG cultivar 'Grasslands Samson' maintained high growth under water deficit and warm conditions with reduced depletion of soil moisture (indicating enhanced WUE), while data of (He, 2016) linked more negative OP to drought tolerance of PRG and tall fescue plants in a rainout shelter in the field, suggesting the need for further investigation.

It has been found that photosynthetic enzymes of C4 plant species are less sensitive to drought (i.e. PEP carboxylase), which may lead to improved WUE compared to that of C3 plant species together with their unique leaf anatomy (Flexas et al., 2012). Also, C4 plants do not exhibit photorespiration and this in turn increases the efficiency of gas exchange and net assimilation under stressed conditions (Ghannoum, 2009). Plant species with crassulacean acid metabolism (CAM) have originally evolved to avoid high evaporative water loss through the stomata opening at night (Yahia et al., 2019). Moreover, plants with CAM or C4 photosynthetic pathway are able to significantly improve plant WUE through the involvement of pre-fixation of CO_2 into an alternative photosynthetic enzyme (i.e. PEP carboxylase) prior to the usual carbon fixation via Rubisco and that regulatory mechanism may synchronize photosynthetic reactions while minimising energy and water loss under stress (Ferrari & Freschi, 2019). However, several authors have argued against selecting any crop plant for improved WUE because documented information on genotypic differences in WUE within and among plant groups (i.e. C3, C4 or CAM) are incomplete in the literature (Angus & Van Herwaarden, 2001; Flexas et al., 2012; Ludlow & Muchow, 1990; Tanner & Sinclair, 1983). Hence, WUE has attracted little attention in C3, C4, or CAM plants in the literature as a useful selection criterion (Attia et al., 2015; Blum, 2005; Blum, 2009; de Almeida Silva et al., 2012; Tuberosa, 2012; Ullaha et al., 2019). Considering the drought-susceptible nature of C3 species compared to the other two plant types, it is worth exploring

drought-response patterns that contribute to high WUE of C3 elite plants, considering the increasing occurrence of drought, mentioned above.

2.2.4.2 Leaf water relations traits

In the past, plant drought tolerance was measured based on soil moisture characteristics and then focus moved to measurements of leaf water status (Kramer, 1988). Plant-specific water relations traits relevant to evaluation of drought tolerance include: leaf water content, cell turgor, and OP of the cell sap. These traits are altered in plant tissues under stress conditions (Hsiao et al., 1976; Passioura, 1982). Water potential (WP) is the main driving force of plant water relations, and is defined as the potential energy per unit mass of water with reference to pure water at zero potential at 25°C and at atmospheric pressure (Chavarria & dos Santos, 2012; Passioura, 1982; Taiz & Zeiger, 2010).

Water moves spontaneously from spaces or cells with high WP to that with low WP (Kirkham, 2005; Kramer & Boyer, 1995; Lambers & Oliveira, 2019). Accordingly, in the soil-plant-atmosphere continuum (SPAC), water moves from soil (–0.01 MPa) into the plant roots (–0.1 MPa), moves through the xylem sap of the stem (–1 MPa) up to leaves (–10 MPa) and then, evaporates from stomatal pores to the atmosphere (–100 MPa), through a WP gradient (Kirkham, 2005; Kramer & Boyer, 1995). Thus, plant's metabolic activities that are entirely based on the transport of water within the plant system mainly depend on the differences in hydrostatic pressure within SPAC. In addition, differences in vapour pressure (or relative humidity; RH) and osmotic potential (i.e. the amount of dissolved osmotic solutes in two compartments separated by a semipermeable membrane) are also highly influential for the plant water uptake from the soil (Kirkham, 2005; Lambers & Oliveira, 2019), water movements within the plant (i.e. distribution of water from the xylem to plant cells) (Chavarria & dos Santos, 2012; Nonami & Boyer, 1993), and transpiration of plant water to the atmosphere where the WP is very low (Lambers & Oliveira, 2019). For example, leaf expansion of water stressed plants occurs more at night than during the day because plants are less subject to stomatal transpiration as a result of low WP gradient between soil WP and atmospheric vapour pressure deficit at night (i.e. cooler temperatures and higher atmospheric RH) (Pantin et al., 2011).

Plant WP represents all the water pressure components acting on the cells of a given leaf, that it is the sum of leaf water potential (LWP), OP, and hydrostatic pressure exerted from cell walls or the turgor potential (TP) (Chavarria & dos Santos, 2012; Kirkham, 2005; Kramer & Boyer, 1995; Lambers & Oliveira, 2019). The main component of soil WP is the matric potential which is a component of WP due to the adhesion of water molecules to non-dissolved structures of soil particles (Klute, 1986). A smaller component is the gravitational potential (i.e. the component of the WP determined by downward pull of water by gravity; this component is usually ignored in plant research, except in tall trees). A third factor to be considered is soil salinity, and in saline soils OP of the soil solution also becomes an important component (Kirkham, 2005; Passioura, 1982).

In most plants, OP is the major component which can actively be adjusted with a passive balance between OP and TP to modify WP of plant cells (Korolev et al., 2000; Lambers & Oliveira, 2019). As the soil dries, causing soil WP to decline, plant WP is adjusted through the accumulation of osmotically active compounds or the reduction of OP (i.e. more negative OP) (Blum, 2017; Lambers & Oliveira, 2019). In particular, drought tolerant plant genotypes are capable of maintaining turgor through increased solute accumulation under severe drought conditions (Blum, 2017). Therefore, OA at low leaf WP, that avoids cell damage under imposed drought, is recognised as a beneficial drought adaption strategy of drought-tolerant plants (Abid et al., 2018; Babu et al., 1999; Begg, 1980; Blum, 2017; Cyriac et al., 2018; Munns, 1988).

Under optimum growing conditions, TP in living cells is always positive as physiologically active cells maintain a positive hydrostatic pressure (i.e. high turgidity) (Korolev et al., 2000; Lambers & Oliveira, 2019). Plant TP is generally reduced as drought stress develops. The magnitude of TP reduction depends on the degree of accumulation of osmotic solutes in cell vacuoles and also, the properties of cell wall elasticity (Kramer & Boyer, 1995; Lambers & Oliveira, 2019). However, osmotic and elastic adjustments are two independent drought responses that contribute to the dehydration tolerance of plants in response to water deficit (Lambers & Oliveira, 2019).

Cell wall elasticity allows plant cells to increase in volume (with increasing solute accumulation) or decrease their WP (i.e. more negative WP) as water gains energy from absorption of the elastic forces of the cell wall that is being tightened by the more negative OP (Lambers & Oliveira, 2019). These plant cell responses occur at different rates under stress, depending on the magnitude of the bulk elasticity modulus (Mitchell et al., 2008; Ngugi et al., 2003; Thomas, 1987) in plant cells until the turgor-loss point is reached (Lambers & Oliveira, 2019). In particular, plants can either use higher cell wall elasticity (i.e. lower bulk elasticity modulus) to store water at less negative OP as a strategy to overcome fluctuating water supply, or they can use more negative OP with less elastic (i.e. higher bulk elasticity modulus) cells to create a more powerful soil water withdrawal (Lambers & Oliveira, 2019). However, under severe water deficit conditions, cell wall elasticity is highly unlikely to contribute sufficient adjustment to maintain cell turgor and a large negative hydrostatic pressure arises within a plant's xylem vessels under rapid transpiration leading to a suction tension and in extreme cases air embolism and loss of conductivity (Lambers & Oliveira, 2019).

2.2.4.2.1 Leaf water potential

Information on plant water stress levels as characterised by LWP has been directly used in scheduling irrigation budgets of field crops (Howell et al., 2012). Also, LWP has long been employed as a selection criterion for screening genotypes for drought tolerance (Abid et al., 2018; Blum, 2005; Chaves, 2002; Jongdee et al., 2002; Yan et al., 2017) and genotypic variation in LWP has also been employed as a selection criterion in crop improvement programs (Avramova et al., 2019; Blum, 2005; Manavalan et al., 2009).

Diurnal variations in LWP of field-grown crops in response to varying evapo-transpirational demand throughout the day have been evaluated in several experiments (Acevedo et al., 1979; Chaves, 2002). Accordingly, LWP varies through the day, with the least negative value (the highest LWP) occurring predawn and the most negative value (the lowest LWP) occurring around solar noon with a gradual fall from the predawn value to the midday value and conversely, a gradual increase from midday to the maximum at night predawn the next day (Jones et al., 1980a). Therefore, predawn and in the middle of the day are considered two critical

times to measure LWP to define a water stress level of a given plant, the former indicating the 'resting' LWP, and the latter the LWP under full transpiration load (Ritchie & Hinckley, 1975; Williams et al., 2012). Otherwise, LWP measurements need to be timed within the day to capture one state or the other, for valid comparisons between plants. Usually, psychrometric and pressure chamber techniques were used to quantify the predawn LWP in stressed plants in experiments (Turner, 1981).

Leaf water potential fluctuations are closely associated with the variations in hydrostatic pressure and OP of plant cells because negative OP and positive hydrostatic pressure jointly keep plant cells alive, turgid, and physiologically active at very low LWP levels or under stress conditions (Korolev et al., 2000; Lambers & Oliveira, 2019).

2.2.4.2.2 Osmotic potential

Osmotic potential is a component of WP that arises from the effect of dissolved solutes in plant cells (Lambers et al., 2008). The OP always has a negative value because water tends to move across a semipermeable membrane from pure water into water containing solutes (Lambers & Oliveira, 2019). The negativity of OP increases as the amount of solutes dissolved per volume of water increases in plant cells (Abid et al., 2018; Begg, 1980; Blum, 2017; Lambers & Oliveira, 2019; Passioura, 1982; Serraj & Sinclair, 2002). Refractometric, cyroscopic, psychometric and, pressure chamber techniques are generally used to measure OP in plants (Turner, 1981).

As soil moisture stress develops, causing soil WP to decline, OP becomes more negative as osmotically active compounds tend to accumulate in cells to adjust cell turgidity (Lambers & Oliveira, 2019; Serraj & Sinclair, 2002). It was also found that drought-tolerant plant genotypes exhibit more negative OP than drought-susceptible ones (Abid et al., 2018; Bothe et al., 2018; Matin et al., 1989; Simane et al., 1993). In PRG, improved summer performance was often associated with more negative OP when pasture plants were grown under moderate to severe moisture stress conditions (Cyriac et al., 2018; Volaire et al., 2009; Volaire et al., 1998).

Osmotic solutes (mostly inorganic ions and organic acids) are mainly accumulated in the vacuole of plant cells (i.e. vacuole constitutes most of the volume of the plant cell) and control the activity of cytoplasmic enzymes under stress conditions (Lambers & Oliveira, 2019). Primary osmotic compounds include; water soluble carbohydrates (e.g. sucrose, trehalose, glucose, fructose, fructans etc.) or sugar alcohols (e.g. sorbitol), amino acids (e.g. proline, aspartic acid, glutamic acid), methylated quaternary ammonium compounds (e.g. glycine betaine), cyclitols (e.g. mannitol), and inorganic ions (Chaves et al., 2003; Farooq et al., 2009). They also stimulate the synthesis of other compatible solutes that aid cellular mechanisms in the cytoplasm, that seldom incur a cost to the cell metabolism (Blum, 2017; Lambers & Oliveira, 2019). Thus, osmotic compounds maintain cell turgor pressure in order to maintain cellular membrane stability and metabolic functions that may eventually delay plant dehydration (Farooq et al., 2009).

Amongst others, accumulation of glycine betaine is considered to be the key to cellular osmoregulation in field crops under stress conditions (Amiard et al., 2003; Arab et al., 2019; Grumet & Hanson, 1986; Lopez et al., 2019; Manavalan et al., 2009; Xing & Rajashekar, 1999). Exogenous application of glycine betaine as an artificial osmoprotectant for grass species has been trialled in several drought experiments (Blum & Ebercon, 1981; Burgess & Huang, 2014; Gan et al., 2018; Zhang et al., 2014). For example, Gan et al. (2018) reported that glycine betaine pre-treatment may avoid the deleterious effects of water stress through increased turf growth, quality, and chlorophyll content and restrained oxidative stress in creeping bentgrass under imposed drought. However, information describing natural accumulation of glycine betaine in temperate grasses in response to drought is almost absent from the literature.

Under drought stress, levels of water soluble carbohydrates (WSC) and sugar alcohols have been found to increase rapidly in field crops (Abid et al., 2018; Amiard et al., 2003; Chaves, 2002; Sánchez et al., 1998) and also in *Lolium* species (Humphreys, 1989a; 1989b;1989c; Richardson et al., 1992; Robins & Alan Lovatt, 2016; Sánchez et al., 1998; Sandrin et al., 2006; Thomas, 1990; Volaire et al., 1998). In particular, fructan, as a high molecular weight WSC (3–8 degree of polymerization) and a dominant osmotic compound, was found in considerable

amounts in the stubble of PRG under drought (Amiard et al., 2003; Evans et al., 2016; Pollock & Jones, 1979; Rogers et al., 2019; Shahidi et al., 2017). It was speculated that fructans may prevent cell membrane damage in PRG under drought (Amiard et al., 2003; Hou et al., 2018; Pollock & Jones, 1979; Rogers et al., 2019; Shahidi et al., 2017; Volaire et al., 1998).

As soil drought develops, concentrations of soluble proteins usually display a gradual decrease. Free amino acids and proline concentrations tend to be increased in several crops (Abid et al., 2018). For example, proline was found in excess amounts in several plants (Bates et al., 1973; Delauney & Verma, 1993; Szabados & Savoure, 2010) including PRG in response to moisture stress (He, 2016; Kemble & Macpherson, 1954; Thomas, 1990; Volaire et al., 1998). It was found that proline plays a role as a hydroxyl radical scavenger to avoid plant cell damage in plants under severe drought conditions (Lambers & Oliveira, 2019). Apart from key organic solutes, inorganic ion accumulation contributes greatly to the osmoregulation in temperate grass species even in comparatively severe drought conditions (Cyriac et al., 2018; Hsiao et al., 1976; Jiang & Huang, 2001c; Sanders & Arndt, 2012; Thomas, 1986, 1990).

The decrease in OP and the simultaneous increase of hydrostatic pressure in the cell sap due to solute accumulation in the cytoplasm are collectively referred to as ‘osmotic adjustment’ (OA) in plants (Begg, 1980; Munns, 1988; Turner, 1986a). Plants that are capable of exhibiting OA tend to exhibit more negative OP at very low LWP levels and that response is directly linked to high plant tissue relative water content (RWC) (Jiang et al., 2009; Matin et al., 1989; Sanders & Arndt, 2012). Thus, there are several methods of estimating OA in field crop species using OP and RWC measurements recorded at several stages of a drought cycle (i.e. pre-drought and drought and rehydration at post-drought) but, with some limitations in each method (Babu et al., 1999). For example, RWC measurements were directly being used for OA estimations in PRG together with OP measurements, particularly when the well-watered treatment or post-drought rehydration step was absent in simulated drought experiments (Cyriac et al., 2018).

OA may assist plants to reduce water loss through stomatal adjustments followed by controlled photosynthesis rate and the postponement of leaf rolling, wilting, and

senescence (Johnson & Asay, 1993; Ludlow & Muchow, 1990; Morgan & Condon, 1986; Morgan, 1984; Sanders & Arndt, 2012; Thomas, 1990). OA also maintains water uptake from drying soil with a greater 'pulling power' of the intracellular solutes for water. OA appears to be associated with the stimulation of deep and/or dense root growth under drought (Blum, 1996; Blum, 2005; Blum, 2017; Morgan, 1995; Morgan & Condon, 1986; Sanders & Arndt, 2012; Serraj & Sinclair, 2002; Turner, 1986a; Zlatev & Lidon, 2012). The trait linkage between more negative OP and deep rootedness has been well-documented in many crops including PRG as a beneficial drought response (Cyriac et al., 2018; Johnson & Asay, 1993; Pollock & Jones, 1979; Rogers et al., 2019; Shahidi et al., 2017; Thomas, 1990; Thomas & Evans, 1989; Turner et al., 2008; Volaire et al., 1998). However, its importance for yield gain is disputed with respect to many crop plants because it is assumed that the accumulation of osmolytes may incur an additional metabolic cost, thus inhibiting plant growth under drought (Blum, 2005; Blum, 2017; Blum & Tuberosa, 2018). According to Lambers & Oliveira (2019), the extent of the expected yield loss, growth instability, and disrupted net assimilation due to the accumulation of osmolytes under drought may depend on the major type of solute/s accumulated in plant cells in response to drought.

Some solutes may have unforeseen negative effects on cell metabolism and key physiological mechanisms (e.g. glycine betaine) (Smirnoff & Cumbes, 1989) while the effects of most solutes are negligible (i.e. compatible solutes namely sorbitol, mannitol, and proline) (Blum, 2017; Lambers & Oliveira, 2019). Thomas & Evans (1989) asserted that OA is a less important drought response for the yield advantage in PRG. However, the respective experiment was carried out in sand-based media (with low WHC) under intermittently imposed mild to moderate water deficit conditions (LWP: –0.7 to –0.8 MPa, OP: –1.7 to –1.9 MPa) for less than a month (Thomas & Evans, 1989). This indicates that the rapid dry-down of potting media did not allow time for a gradual intracellular physiological adjustment, or less severe moisture deficit provided in the experiment might not have fully stimulated drought responses. However, a recent study conducted with multiple drought cycles suggested that OA, representing a beneficial dehydration avoidance response, may be A key determinant of PRG summer performance (Cyriac et al., 2018). These superficially differing perspectives of water relation trait responses on the yield gain

and survival of PRG in drought challenge events highlight the importance of further research.

2.2.4.2.3 Leaf relative water content

Relative leaf water content or the relative leaf tissue turgidity (often measured for leaf tissue) is a measure of the leaf tissue actual water content or the level of leaf hydration relative to its maximum water holding capacity at full turgidity, shown in the following equation:

$$RWC\% = ((FW-DW)/TW-DW) \times 100$$

Where, FW is the fresh leaf weight, DW is the oven dried weight at 80°C for 24 hours and, TW is the full turgid weight after soaking leaf tissue in distilled water for minimum of four hours (in the dark to avoid weight change from on-going photosynthesis, and avoiding high temperature to minimise respiration) (Barrs & Weatherly, 1962; Turner, 1981).

As soil drought develops, daily plant water uptake falls below the transpirational demand, leading to a significant decrease in LWP (Section 2.2.5.2.1). Despite the fact that low LWP generally results in low RWC, it has been found that drought tolerant genotypes can withstand plant dehydration through the maintenance of high and stable RWC that is associated with more negative OP at low LWP (Section 2.2.5.2.2) (Abid et al., 2018; Blum, 2005; Chaves, 2002; Marček et al., 2019). For instance, drought tolerant tall fescue genotypes showed a slower rate of decline in RWC than drought susceptible genotypes at low LWP despite the general reduction observed in both RWC and LWP of test forage grass species under imposed drought (i.e. LWP and RWC values of two tall fescue cultivars simultaneously dropped from −5 bars to −20 bars and 90% to 50%, respectively, in a 10–12 days of drought) (Huang et al., 1998a).

Generally, drought tolerant plant genotypes maintain a stable RWC under drought, and therefore there is comparatively less genotypic variation for RWC in elite germplasm lines (Matin et al., 1989; Teulat et al., 1997; Wang & Bughrara, 2008). For that reason, RWC has been considered as a covariate to adjust the genotypic values of OP or stomatal conductance in drought-challenged plants, in order to

facilitate screening of genotypes for drought tolerance at a constant plant RWC (i.e.70% level RWC for grain crops) (Acevedo et al., 1979; James et al., 2008; Manavalan et al., 2009).

Precise measurements of plant water status, including RWC, are important to assess the relative capacity of OA in dehydrated plants under imposed drought (Merah, 2001; Sanders & Arndt, 2012; Sinclair & Ludlow, 1986; Taiz & Zeiger, 2010; Teulat et al., 2003). Also, RWC can serve as an indicator of physiological injury, permanent physiological damage, and incipient death of plant cells that can occur at a critical RWC (species-specific and tissue-specific) under severe drought conditions (Chai et al., 2010; James et al., 2008; Sinclair & Ludlow, 1986; Taiz & Zeiger, 2010; Wang & Huang, 2004).

2.2.4.3 Root adaptations

Root behaviour is one of the highly researched drought tolerance traits, as a deeper or more prolific rooting pattern can ensure plant water uptake from dehydrating soil layers to maximise plant access to scarce water during drought a period (Bonos et al., 2004; Comas et al., 2013; Serraj & Sinclair, 2002; Sinclair & Muchow, 2001; Thomas, 1997).

In previous research, the most dramatic reductions in the percentage of roots, due to damage in the cortex and root hairs, reportedly occurred only at a soil matric potential of –3 to –4 MPa while the death of root tips in both main roots and most lateral roots reportedly occurred when soil matric potential was below –10 MPa (Jupp & Newman, 1987). In PRG, the number of cells in both the topmost roots and lateral roots exhibited a significant decrease under a progressively developing drought challenge and root growth completely ceased under prolonged and severe drought events (Bonos et al., 2004; Volaire et al., 1998).

Roots are generally less vulnerable to drought stress than shoots (Blum, 2005). Hence, root to shoot ratio (RSR) increases under drought as a greater proportion of assimilates tends to be diverted to roots in response to the stress (Blum, 2005; Crush et al., 2004; Huang & Fry, 1998b; O'toole & Bland, 1987; Passioura, 1983). Morgan (1984) noted that in the absence of leaf OA may lead to increased RSR as a common drought-adaptive response in plants and that increases water availability to the roots

under drought. Also, a recent study found that increase in RSR in response to drought stress is a result of the accumulation of high levels of soluble sugars in roots from the increase in leaf sucrose-phosphate synthase and root invertase enzyme activities in leaves that enable the transport of sugars from leaves to roots (Xu et al., 2015). In sunflower plants, abscisic acid has also been identified as a multi-functional plant growth regulator, the levels of which are elevated under drought stress, assisting translocation of assimilates to the roots (Rauf & Sadaqat, 2007). Consistent with this fact, one possible reason found for deep rootedness is the differential sensitivity of leaves and roots to endogenous ABA (abscisic acid) production in addition to different levels of OA or turgor maintenance found in shoot and root cells of studied plant species (Sharp & Davies, 1989).

Mwenye et al. (2018) found that drought tolerance properties of soybean cultivars were associated with increased RSR. However, most literature placed less importance on RSR for drought tolerance of temperate grasses (Bonos et al., 2004; Chaves & Oliveira, 2004; White & Snow, 2012). One reason for this difference in opinion was that the deep rootedness was seen as more important than RSR to pasture plant drought tolerance (White & Snow, 2012). It was stated that in a drought, a very low proportion of functional roots could be seen in the uppermost part of the grass root system, and that the upper part of the root system contributed the highest weight to RSR under conditions tested, compared to that of the deeper root fraction (White & Snow, 2012).

Deep root production for increased water uptake during drought has been discussed as a beneficial drought response in many crops (Abid et al., 2018; Blum, 2005; Chaves, 2002; Condon, 2020; Ekanayake et al., 1985; Hund et al., 2009; Lopes & Reynolds, 2010; Manavalan et al., 2009; Matsui & Singh, 2003; Passioura, 1983; Vadez, 2014) including PRG (Bonos et al., 2004; Bothe et al., 2018; Crush et al., 2002; Johnson & Asay, 1993; Volaire et al., 1998). Osmotic potential and deep rootedness were found to be correlated in drought tolerant crops (Abid et al., 2018; Blum, 1996; Blum, 2005; Chaves, 2002; Manavalan et al., 2009).

Deep root development is a complex trait as various root morphological characters contribute to it (Kato et al., 2006), including vertical or lateral root distribution (Cooper & Ferguson, 1964; Grieder et al., 2014; Hund et al., 2009), root angle (Kato

et al., 2006; Mambani & Lal, 1983), root thickness (Tsuji et al., 2005; Yambao et al., 1992), number of root hairs (Huang & Fry, 1998; Jupp & Newman, 1987), root viability (Bonos et al., 2004; Chaves & Oliveira, 2004; Wedderburn et al., 2010) (Huang et al., 1997), root surface (Comas et al., 2013), and root length or root length density (Asch et al., 2005; Henry et al., 2011; Hund et al., 2009). Root biomass, as a representative of most root characteristics, is often considered to be a feasible way to envisage the root growth at different soil depths under drought (Huang et al., 1997; Lopes & Reynolds, 2010; Wedderburn et al., 2010). Knowledge of the role of each root characteristic in the function of the root system under drought may facilitate breeding of grass species for drought tolerance (Huang et al., 1997). For example, it was found that thinner and lighter roots generally facilitate plant water uptake but, thicker and heavier roots resist water uptake under moisture limiting conditions (Carvalho & Foulkes, 2018).

Within-population variation for deep rootedness in drought tolerance has been extensively discussed in several crops (Bolaños et al., 1993; Dalal et al., 2017; Gowda et al., 2011; Nguyen et al., 1997; Ribaut et al., 2009) including PRG (Cui et al., 2015) under imposed drought. Genotypic diversity that exists in PRG populations for deep rooting behaviour may be a great resource for improving PRG germplasm lines for drought tolerance (Sokolovic et al., 2013; Turner et al., 2010). However, the questions remain: to what extent is such a root feature involved in beneficial drought-response patterns that aid either or both plant production and survival, and what is its contribution to EUW or WUE (Blum, 2005; Chaves, 2002; Dalal et al., 2017).

2.2.4.4 Stomatal adaptations

Abscisic acid generated in the roots is one of the primary signals observed in plants that are exposed to drought stress (Fan et al., 2008; Schroeder et al., 2001). The ABA signal is transferred from roots to the stomatal guard cells of leaves via the xylem and there triggers ion fluxes between guard cells and the adjacent cells causing guard cell shrinkage and stomatal closure in response to drought (Schroeder et al., 2001). Under unfavourable conditions, including drought, stomatal closure restricts water loss at the expense of CO_2 intake (Cornic, 2000), This strategy has associated secondary effects such as increased canopy temperature or canopy

temperature depression (Kimball & Bernacchi, 2006), reduced uptake of nutrients (Renkema et al., 2012; Yingjajaval, 2012), and increased photorespiration (Wingler et al., 1999). However, during the early stages of soil drought, the size of the stomatal aperture is primarily regulated by changes in air humidity and air temperature (Aliniaeifard et al., 2014; Araújo et al., 2011; Feller, 2006; Hall et al., 1975; Hofstra & Hesketh, 1969; Morison & Gifford, 1983; Woledge et al., 1989).

For field crops, stomatal conductance (SC) is considered to be a major determinant of yield under stress conditions (Araus et al., 2002; Faralli et al., 2019; Khan et al., 2010; Laffray & Louguet, 1990; Quarrie & Jones, 1979). The high sensitivity of stomata to stress conditions causes adverse impacts on a plant's photosynthesis, growth, and yield under drought (Araghi & Assad, 1998; Blum, 1996, 2009; Easlon et al., 2014; Flexas et al., 2002; Lafitte et al., 2006; Medrano et al., 2002; Motzo et al., 2013; Shahinnia et al., 2016; Turner, 1986a). Hence, SC has long been used as a selection criterion when selecting field crops for drought tolerance (Faralli et al., 2019; Rebetzke et al., 2013; Silva-Pérez et al., 2020). Stomatal frequency or distribution, and size or dimensions of stomata are also potential selection criteria in crop selection programs (Flexas et al., 2002; Jones, 1976, 1977; Khazaie et al., 2011; Lawson & Blatt, 2014; Mishra, 1997; Venora & Calcagno, 1991) including PRG selection experiments (Wilson, 1971, 1972). Moreover, certain indirect trait measurements of SC (i.e. canopy temperature, succulence index, wilting score, leaf reflectance, epidermal ridging, presence of leaf hair or trichomes) have also been employed in screening plants for drought tolerance, particularly at early growth stages (Ober et al., 2005; Rebetzke et al., 2013). In temperate grass species, the ability of stomata to control water loss in response to drought was found to be an important trait (Jones et al., 1980, 1980b; Shahidi et al., 2017).

Irrespective of the soil moisture availability, SC in cool-season grass species is associated with other species-specific trait responses such as leaf turgor maintenance that is supposed to be mediated by root water uptake capacity (i.e. deep rootedness) of each species (Thomas, 1986). For example, SC of cocksfoot and PRG showed significant differences in drought responses in such a way that differences in SC were attributed to leaf water status and rooting characteristics in the two grass species under the conditions tested (i.e. SC of deep-rooting cocksfoot was 33%

greater than that of shallow-rooting PRG under well-watered conditions, while it was 25% lower than that of deep-rooting PRG under moisture stress) (Thomas, 1986). Similarly, tall fescue genotypes showed higher SC and evapotranspiration rates than PRG genotypes in both well-watered and drought conditions and that was linked to a differential rooting properties. Tall fescue had inherently deep and dense rooting traits that led to water uptake from deeper soil layers to keep assimilation undisturbed (Jiang & Huang, 2001b). Hence SC, OP, RWC, LWP, and deep rootedness were found to be highly interrelated in previous research (Abid et al., 2018; Blum, 2005; Chaves, 2002; Cyriac et al., 2018; Pimentel et al., 1999; Richards, 1996; Thomas, 1990; Thomas & Evans, 1989; Thomas & Evans, 1991; Thomas & James, 1999). Moreover, OP (with RWC and LWP traits) has been considered to be a proxy measure for SC in some crop selection programs (Ashraf & O'Leary, 1996; Ober et al., 2005). In addition, the sensitivity of stomatal movements to drought was found to be influenced by plant growth stage. For example, Thomas & Evans (1989) found that the rate of stomatal closure was slower during the flowering stage than during vegetative growth of PRG under imposed drought. Besides plant physiological and phenological aspects, stomatal movements are regulated by a range of environmental factors such as light intensity, air humidity, atmospheric CO_2 concentration, and temperature (Araújo et al., 2011).

In early research, the benefit of plant water retention due to stomatal closure was speculated to outweigh the negative effects of stomatal restrictions on net assimilation under moisture stress (McCree & Richardson, 1987). It was later found that the initial gains through a decline in transpiration (or water vapour deficit change) from stomatal closure under drought is most likely to be outweighed by its side effects (e.g. increased canopy temperature) in addition to the main adverse effect (i.e. reduced CO_2 intake or net assimilation) (Condon, 2020; Medina & Gilbert, 2015). For plant breeding purposes, recent research focused more on the rate of CO_2 diffusion from sub-stomatal cavities to the sites of carboxylation in the chloroplast (i.e. mesophyll conductance, MC) than that of SC (Condon, 2020; Flexas et al., 2016). It has been found that an enhanced rate of MC can result in increased photosynthesis and WUEi despite the adverse consequences of stomatal closure on the net assimilation under stress conditions (Flexas et al., 2016; Ouyang et al., 2017; Tomeo & Rosenthal, 2017; Zait & Schwartz, 2018; Zhou et al., 2019). However, the

inconsistent relationship found between MC and SC in previous research slowed down the straight forward decision making on which trait to be more focused on for plant breeding purposes (Condon, 2020; Tomeo & Rosenthal, 2017). Ouyang et al. (2017) suggested that modification of stomatal traits may be better than focusing on leaf conductance (both SC and MC) for breeding rice plants for drought tolerance (Ouyang et al., 2017). Apparently, conflicting evidence and opinion about physiological trade-offs with respect to stomatal closure has restricted confidence that incorporating stomatal characteristics into cool-season grass cultivar breeding for drought tolerance could provide gains. Also, the difficulty in obtaining consistent stomatal conductance measurements in the field in early research has aggravated this concern further (Johnson & Asay, 1993; Jones, 1979; Jones et al., 1980a, 1980b; Ludlow & Muchow, 1990). However, Condon (2020) suggested that selecting crops for a combination of traits may resolve such issues in crop improvement programs.

2.2.4.5 Drought effects on photosynthetic gas exchange

2.2.4.5.1 Stomatal & non-stomatal limitations to photosynthesis

Photosynthesis, together with cell growth, is among the primary physiological processes that are adversely affected by water stress (Flexas et al., 2002; Pinheiro & Chaves, 2011; Zlatev & Yordanov, 2004). The drought-induced changes in the mechanisms of photosynthesis or gas exchange characteristics are complex and species or genotype dependant (Chaves, 2002; Chaves et al., 2009; Lauteri et al., 1997). The complex structure of the photosynthesis mechanism involves photosynthetic pigments, photosystems (PS) I and II, the electron transport system, and CO_2 reduction pathways other than the gas exchange through the leaf conductance. A drought effect on one single component may reduce the overall photosynthetic capacity of a plant (Ashraf & Harris, 2013; Chaves, 2002). Hence, effects of drought in restricting photosynthesis can be both direct and indirect (Berry & Downton, 1982; Chaves, 1991; Flexas et al., 2004; Flexas et al., 2002; Pinheiro & Chaves, 2011).

Decreased CO_2 intake caused by reversible diffusion limitations arising from stomatal closure (Berry & Downton, 1982; Cornic & Massacci, 1996; Flexas et al., 2004; Flexas et al., 2007) (as described in the section 2.5.4 above) and alterations in

photosynthetic metabolism (Cornic & Massacci, 1996; Flexas et al., 2002; Lawlor & Cornic, 2002) are direct responses to mild or moderate drought. According to Cornic & Massacci (1996), this CO_2 drop in the chloroplast may primarily cause: (i) a decrease in photochemical yield of open PSII centres and a simultaneous increase of thermal dissipation of the excitons trapped in PS units; (ii) a decline in the activity of primary photosynthetic enzymes, and (iii) an increase in the activity of the Ribulose 1,5-bisphosphate oxygenase that accelerates photorespiration and eliminates the available energy and oxygen levels, in the absence of adequate CO_2 concentrations in photosynthetic sites. Consequently, the natural balance in the production of reactive oxygen species in plant cells can be disturbed causing further damage to the photosynthetic apparatus. Such indirect responses or secondary effects of photosynthesis may cause severe damage to the leaf photosynthetic capacity, for example through oxidative stress (Chaves et al., 2009; Flexas et al., 2002; Ort, 2001) that are usually associated with multiple or severe stress conditions (Chaves & Oliveira, 2004; Chaves et al., 2009).

Under mild to moderate drought conditions, reduced MC may result in significantly reduced CO_2 concentration in chloroplasts relative to that in sub-stomatal cavities, so leading to limited photosynthesis as a non-stomatal limitation (Flexas et al., 2016; Flexas et al., 2007; Grassi & Magnani, 2005; Ouyang et al., 2017; Warren, 2007). However, under severe drought, reduced photosynthetic rate is mainly caused by various types of non-stomatal limitations and subsequently, photosynthesis cannot be completely recovered even by increasing internal CO_2 concentration when the drought is alleviated (Flexas et al., 2002; Grassi & Magnani, 2005; Signarbieux & Feller, 2011). Such non-stomatal limitations include reduced ATP supply as a consequence of loss of ATP synthase activity (Tezara et al., 1999), reduced total chlorophyll ‘b’ content (Moran et al., 1994; Zuily-Fodil et al., 1990) and, decreased levels of primary photosynthetic enzymes (Bayramov et al., 2010). The production of unbalanced amounts of reactive oxygen species in plant cells such as superoxide radicals, hydroxyl radicals, hydrogen peroxide etc. (i.e. by-products of aerobic metabolism of chloroplasts and mitochondria in plant cells) is considered to be the most damaging non-stomatal limitation to photosynthesis in abiotic stress (Gill & Tuteja, 2010; Rodriguez & Redman, 2005; Sharma et al., 2012). This phenomenon causes oxidative damage of organic molecules such as proteins, lipids,

carbohydrates, and DNA of plant cells compromising cell membrane and cellular functions leading to cell death (Flexas et al., 2004; Flexas et al., 2002; Sharma et al., 2012).

2.2.4.5.2 Methods for quantification of irreversible cell damage induced by non-stomatal limitations of photosynthesis

Drought impacts on the plant metabolism become more complex and irreversible as the intensity of drought develops with time. Major irreversible cell and tissue level damage includes oxidative damage, lipid peroxidation, cell membrane failure, cell leakage, and cell death, all of which are primarily induced by non-stomatal limitations of photosynthesis under severe drought (section 2.5.5.5.1).

Dehydration tolerance is a reflection of plant's ability to maintain the integrity in plant metabolic and physiological activities at both the cell level and whole-plant level under imposed drought (Chaves et al., 2003; Tan & Blake, 1993). Under severe drought stress conditions, quantification of plant drought responses at the cell and tissue levels is of utmost importance when describing the complete picture of any drought damage to the test plant. This may eventually define the plant's ability to tolerate the imposed drought together with common drought responses (i.e. water relations, morpho-physiology, and biochemistry) at the whole-plant level (Zhou et al., 2019).

Malondialdehyde (MDA) is a product of peroxidation of unsaturated fatty acids. Such peroxidation is a damaging intracellular transformation in plants, and a good indicator for quantifying the degree of damage to plant tissues under severe stress (Abid et al., 2018; Fu & Huang, 2001; Marček et al., 2019). For example, MDA concentrations of tall fescue and Kentucky bluegrass leaves were eight times higher under intense drought imposed for 30 days (i.e. severe moisture deficit, 17% FC; high temperature, 35ºC) than in well-watered plants (400 and 50 nmol/g fresh weight, respectively) (Jiang & Huang, 2001a). Also, MDA has been used as an indicator of drought tolerance of field crops (Abid et al., 2018) and PRG (He, 2016). For example, MDA concentration of drought-sensitive wheat genotypes was increased from 69 to 89 µmol/g, but was unchanged in drought-tolerant genotypes under prolonged and severe moisture stress (Hameed et al., 2013).

Electrolyte leakage reflects the degree of damage to cellular membranes in response to severe stress conditions, because the amount of electrolyte leakage is basically a function of plant cell membrane permeability (Agarie et al., 1995; Ahmadizadeh et al., 2011; Tan & Blake, 1993). This also applies to PRG (Huang & Fry, 1998b). Several studies have focused on the species-specific responses in the antioxidant defence system in studied plants, in terms of both non-enzymatic (i.e. ascorbate, glutathione, tocopherol, flavonoids, alkaloids, carotenoids, and free amino acids) and enzymatic antioxidants (i.e. superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and, glutathione peroxidase) that are generally produced in plants to diminish oxidative stress caused by severe stress conditions (Abid et al., 2018; Ahmadizadeh et al., 2011; Baker, 2008; Chaves, 2002; Fu & Huang, 2001; Gomes et al., 2010; Hameed et al., 2013; Manavalan et al., 2009; Rodriguez & Redman, 2005; Zhang et al., 2015).

An integrated approach to the drought tolerance at both the cell or tissue level and whole-plant level was tested in two legume forage species; *Medicago truncatula* and *Sulla carnosa*, where the former was found to be more drought tolerant than the latter (Rouached et al., 2013). This selection approach used multiple traits including biomass, RWC, MDA concentration, and electrolyte leakage (Rouached et al., 2013). In another study, drought tolerance of two PRG cultivars was characterised by the presence of lower MDA and reactive oxygen species concentrations and higher antioxidant and ABA levels together with higher herbage yield compared to that of drought sensitive material (Zhang et al., 2015).

2.5.4.5.3 Chlorophyll fluorescence

Chlorophyll fluorescence (CF) is one of the three mechanisms of dissipation of light energy intercepted by leaves. The re-emitted light is termed CF and is 1–2% of the incident light. The other two mechanisms are photochemical quenching (i.e. light capture by the electron transport chain in the photosynthesis process), and non-photochemical quenching (i.e. heat dissipation) (Maxwell & Johnson, 2000). These three processes occur simultaneously, thus imbalance among them may cause impaired photochemistry leading to photoinhibition (Krause, 1988). For that reason, CF parameters have been considered when screening a given germplasm for drought tolerance (Li et al., 2006; Maxwell & Johnson, 2000). The spectrum of fluorescence

differs from that of the absorbed light, with the peak of fluorescence emission being of longer wavelength than that of the absorbed light.

Chlorophyll fluorescence yield can be quantified by exposing a dark-adapted and a light-adapted leaf to light with a defined wave length and measuring the amount of light that is re-emitted at longer wavelengths in both cases (Figure 2.1). The most common CF parameters include F_o (minimum or zero fluorescence), F_v (variable fluorescence), and F_m (maximum fluorescence) (Maxwell & Johnson, 2000). The maximal quantum yield is estimated from the ratio $(F'_m-F_o)/ F^o_m$ and this entity is often referred as $F_{v,}$ where $F'_{m,}$ F_o, and F^o_m are the maximum fluorescence level of light-adapted leaves, the minimum fluorescence level of dark-adapted leaves, and the maximum fluorescence level of dark-adapted leaves, respectively (Figure 2.1). Hence, the ratio F_v/F_m is the most commonly used parameter for measuring the maximum or potential photochemical efficiency of PSII of dark-adapted leaves (Jiang & Huang, 2001b; Maxwell & Johnson, 2000; Signarbieux & Feller, 2011). Generally, the F_v/F_m ratio is in the range of 0.79−0.84 in dark-adapted and non-stressed plant species (Björkman & Demmig, 1987; Maxwell & Johnson, 2000) Under stressed conditions, the fluorescence yield goes high causing (and as well as heat dissipation or non-photochemical quenching) a decline in photochemical absorption of incipient light (i.e. photochemical quenching) and eventually the photochemical efficiency due to photoinhibition (Krause & Weis, 1991). Thus, stressed plants exhibit lower F_v/F_m values (with higher F_m) which may be used as an indicator of the stress impact on a given plant (Krause & Weis, 1991). For example, the F_v/F_m value of tall fescue plants tested in a simulated drought was significantly reduced when RWC fell to 60% (Huang et al., 1998a). It was also found that F_v/F_m values can be used to determine the degree of stress tolerance of experimental plants (Baker, 2008; Filek, 2006). In a drought experiment, drought-tolerant barley cultivars showed higher F_v/F_m values than that of drought-sensitive barley cultivars (i.e. average F_v/F_m of drought-tolerant and drought-sensitive cultivars were 0.79±0.01 and 0.72±0.11, respectively) (Li et al., 2006). Quantification of these key CF parameters have demonstrated their practical importance when determining the impact of drought stress or drought tolerance in PRG as well, particularly under moderate to severe soil moisture stress conditions (Amalric et al., 1999; Cui et al., 2015; Shahidi et al., 2017). Moreover, genotypic variance observed for CF

parameters in different PRG cultivars and accessions showed the potential of CF parameters to be used as selection criteria in screening a given PRG germplasm for drought tolerance (Dąbrowski et al., 2019; Yu et al., 2013).

The photochemical quenching parameter (qP, where qP = $((F'_m-F_s)/ F'_m-F'_o)$ and the efficiency of PSII (φPSII; where φPSII = $(F'_m-F_t)/F'_m$; F'_m is the maximum fluorescence level of light-adapted leaves and F_t is the steady-state value of fluorescence) are the most important parameters when measuring the proportion of the light absorbed by chlorophyll of leaf tissues that is directly associated with the PSII photochemistry (Genty et al., 1989; Maxwell & Johnson, 2000). The parameter of φPSII can give an accurate measure of the linear electron transport rate (ETR) as an indication of the overall photosynthesis (Maxwell & Johnson, 2000). Photochemical quenching gives an indication of the proportion of open PSII reaction centers during photosynthesis (Maxwell & Johnson, 2000). Dąbrowski et al. (2019) found a significant difference between the drought-sensitive and drought-resistant PRG varieties for φPSII and ETR under in-vitro drought conditions, suggesting such measurements may be important in defining the drought tolerance property of a given PRG population together with gas exchange measurements.

Non-photochemical quenching (NPQ or qN, where qN = $(F^o_m-F'_m)/ F'_m$) of a given plant is linearly related to heat dissipation at the light-adapted state of leaves relative to the dark-adapted state that may range from 0.5–3.5 under stress conditions depending on the species (Maxwell & Johnson, 2000). Hence, this parameter has been widely utilised to explore differences in photoprotection and photoinhibition properties in various plant species under imposed stress conditions (Demmig-Adams et al., 1995; Maxwell & Johnson, 2000).

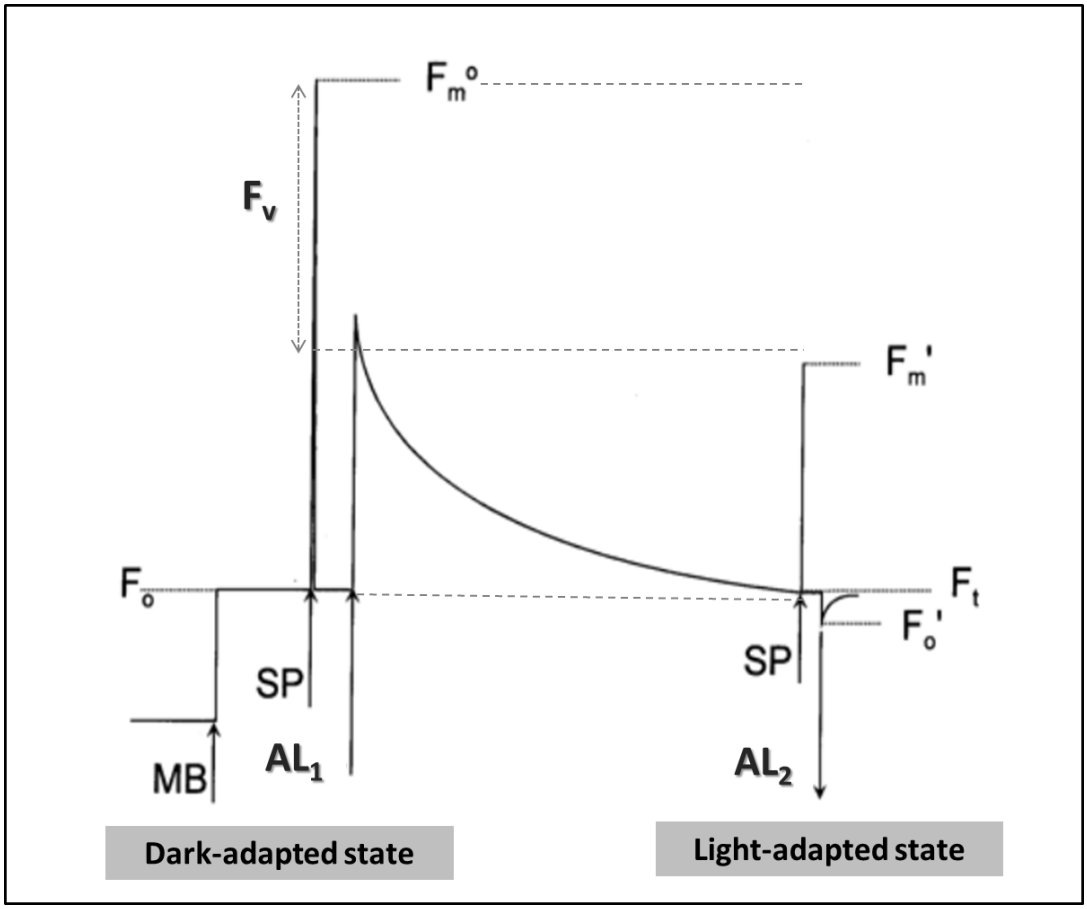

Figure 2.1 An illustration of a typical fluorescence trace (modified from (Maxwell & Johnson, 2000)) (Abbreviations: MB, measuring light; $F_{o,}$ zero fluorescence level of dark-adapted leaves; SP, saturating flash of light; F^{o}_{m}, maximum fluorescence level of dark-adapted leaves; AL_1, actinic light is turned on to drive photosynthesis; F'm, maximum fluorescence level of light-adapted leaves; $F_{v,}$ the level of fluorescence immediately before the second saturating flash and calculated as $(F^{'}_{m}-F_o)/ F^{o}_{m}$; AL_2, actinic light is turned off and far-red light is turned on, $F'_{o,}$ zero level fluorescence 'in the light'; F_t, the steady-state value of fluorescence immediately prior to the flash in the light-adapted state of the leaf).

2.3 Perennial ryegrass breeding for drought tolerance

2.3.1 Trait selection approaches in PRG breeding programmes: Considerations for the enhancement of pasture drought tolerance

According to Acquaah (2012), plant breeders have two basic selection approaches in breeding plants conventionally for drought tolerance, namely indirect selection and direct selection.

Indirect selection refers to the selection of plants for target traits at locations where stress conditions exist but under non-stressed conditions (Acquaah, 2012; Atlin & Frey, 1989). Richards et al. (2002) suggested that it is advisable to screen wheat crops for target traits under non-stress conditions, particularly when the shoot biomass or the harvestable yield is considered to be the main breeding objective. Richards (1996) stated that some of the greatest successes in wheat breeding were achieved by breeding wheat accessions in environments where water is non-limiting. Also, as noted in 2.2.4.4 above, selection of plants under well-watered conditions is ideal. However, similar information is almost absent from the literature describing pasture cultivar selection programs.

In the direct selection approach, which is the most common method of forage grass selection and breeding (Bahmani et al., 2000; Bonos et al., 2004; Ravel & Charmet, 1996; Robins & Alan Lovatt, 2016; Sampoux et al., 2011), plant genotypes are exposed to the relevant stress when selecting them for the target traits (Acquaah, 2012; Kumar et al., 2008).

The direct selection approach targeting improved plant drought tolerance may include the following trait evaluation strategies:

1. Field selection for target traits that may create inconsistent selection pressure from one cycle to the next (Acquaah, 2012; Hill, 1975) (Note: breeders generally conduct multi-environment multi-year trials to assess G×E interactions and extract genetic factors that enhance drought tolerance) (Condon & Richards, 1992; Kang et al., 2004; Ravel & Charmet, 1996),

2. Selection under managed stress environments including glasshouse plot trials and rainout shelter experiments (Acquaah, 2012; de Almeida Silva et al., 2012; Nguyen & Sleper, 1983),

3. Selection based on yield *per se*, even though this is an inefficient approach due to the fact that yield measured under stressed rather than under non-stressed conditions is less heritable (Harmer et al., 2016; Richards et al., 2002) (Note: breeders seek to develop efficient, less expensive, and high throughput and non-conventional approaches for screening large populations

to overcome this problem) (Simeão Resende et al., 2014; Xu et al., 2017; Yates et al., 2019),

4. Selection based on morphological traits that includes shoot and root developmental adjustments to drought: the most common practice (Blum, 2005; Dalal et al., 2017; Kato et al., 2006; Lopes & Reynolds, 2010), and

5. Selection based on assessment of plant water status that is an integral part of the plant metabolism and physiological trait responses (Acquaah, 2012; Arab et al., 2019; Blum, 2005; Edwards et al., 2012; Manavalan et al., 2009).

Plant selection approaches 1, 3, and 4 above have been largely employed in conventional pasture breeding programs in New Zealand for many years (Harmer et al., 2016; Hunt & Easton, 1989; Lee et al., 2012; Stewart, 2006; Williams et al., 2007; Woodfield, 1999). New PRG cultivar development has been using traditional and typical mass selection methods that involve the identification of promising germplasm based on the visual selection of elite individuals for vigour and yield from field nurseries of thousands of single plants, followed by company specific crossing and field plot testing systems leading to eventual cultivar release (Easton et al., 2001; Stewart, 2006). To the author's knowledge, only a few commercial PRG breeding operations actively screen for morpho-physiological traits targeting pasture drought tolerance at the plant genotype level at present. Approaches 2 and 5 above, particularly for PRG, have mostly been used at the experimental level that aimed at better understanding of the differences in drought response patterns between germplasm lines (Hussain, 2013) or the physiology of drought tolerance (He, 2016; Hussain, 2013).

Non-conventional selection approaches (i.e. advanced molecular plant breeding) have already gained attention in New Zealand pasture breeding research (Lee et al., 2010; Parsons et al., 2011; Stewart & Ellison, 2014; Williams et al., 2007). For example, QTL mapping was used in screening PRG for pasture vigour and persistence under imposed moisture stress in New Zealand research in the recent past (Crush et al., 2007; Hatier et al., 2014).

The success of any trait evaluation strategy depends on several factors including the knowledge on different drought types (i.e. terminal or intermittent drought) (Acquaah, 2012; James et al., 2008; Ludlow & Muchow, 1990; Manavalan et al., 2009), the degree of drought stress (i.e. mild, moderate, or severe drought) (Abid et al., 2018; Ludlow & Muchow, 1990), beneficial drought-response trait response for plant drought tolerance (Acquaah, 2012; Blum, 2005; Chaves, 2002; Condon, 2020; Ludlow & Muchow, 1990), and underlying plant physiological principles of plant's drought response (Acquaah, 2012; Passioura, 1976; Passioura & Angus, 2010). Apart from having that knowledge, the consistency of the drought tolerance trait response should be given great attention in plant improvement attempts because once a certain genotype is selected for its performance in one season, it is expected that performance lasts throughout its life cycle or several growing seasons (da Silva et al., 2006). For that reason, Kemp & Culvenor (1994) suggested that it is advisable for pasture breeders to select perennial pasture grasses through a minimum of two-cycles of establishment and regrowth under dry conditions. It is also advisable to expose experimental plants to an adequate duration and intensity of soil drying for facilitating physiological adjustment in plant growth and development to a noticeable level in each drought cycle (Farooq et al., 2009).

Acquaah (2012) asserted that the direct selection is best conducted under controlled environmental conditions where the stress factors occur uniformly and predictably because the micro-environmental variables (i.e. VPD, RH, and temperature), that simultaneously appear in a soil moisture deficit condition and influence the degree of plant stress, are highly variable in field settings. Therefore, this aspect of the environmental effect on the plant drought response is generally quantified as a 'genotype-by-environment interaction (G×E) effect' in plant quantitative genetic studies (Hill, 1977; Hill, 1971). The relative effectiveness of direct or indirect selection depends upon the heritability and genetic correlation estimates of primary and secondary traits in both non-stressed and stressed situations as imposed on the same population under similar experimental conditions (Atlin & Frey, 1989).

2.3.2 Examples of PRG improvement methods used in New Zealand: History and recent trends

Perennial ryegrass was first introduced to New Zealand by British immigrants in the second half of the 19th century (Stewart, 2006). Since then, more material was imported to New Zealand at various times. Locally produced seed from already-established pastures was traded within both islands of the country and a trans-Tasman seed trade also existed in the late 19th century and early 1900s (Hunt & Easton, 1989; Stewart, 2006).

Naturalised PRG germplasm collected from drier parts of New Zealand, mainly Hawkes Bay, was first evaluated by Mr. E.B Levy and Mr. W.M. Davis at the Plant Research station (later named Grasslands Division of the Department of Scientific and Industrial research; DSIR Grasslands) in Palmerston North (Easton, 1983). This collection showed a large within- and among- ecotype variation for selection and was the foundation for subsequent local breeding programs (Stewart, 2006). Realizing the potential contribution from superior strains of old local pastures as mother materials, the Government Seed Certification Scheme was established to safeguard seed multiplication of the elite lines under acceptable standards in the 1920s (Scott, 1980). Consequently, the first PRG strain called "New Zealand pedigree" was introduced after several generations of recurrent selection of the superior Hawkes Bay ecotype that was identified by Levy and Davis in the 1930s, and was later renamed 'Grasslands Ruanui' in 1955 (Armstrong, 1977; Stewart, 2006). Thus, 'Grasslands Ruanui' emerged as the benchmark PRG cultivar in New Zealand. The introduced European germplasm (mostly from Ireland, Ayrshire and, Devon), compared to the local material was winter-dormant (Stewart, 2006). Therefore, one of the early goals of PRG breeding was to improve the winter growth because the winter in New Zealand is milder than that in Europe (Stewart, 2006; Williams et al., 2007).

Increased resistance to crown and stem rust was another of the main objectives in PRG breeding programs in New Zealand (Easton et al., 1989; Lancashire & Latch, 1970; Lee et al., 2012). Hence, improved characters of 'Grasslands Ruanui' included high leaf production, persistence, resistance to crown rust, and high winter and spring yield (Stewart, 2006; Wratt & Smith, 1983).

The next step of PRG breeding was to exploit the high winter growth of Italian ryegrass while moving forward with both the local and exotic material (Stewart, 2006). Thus, previously selected elite PRG genotypes and Italian ryegrass breeding lines were hybridized and a hybrid cultivar H1 was released as a short-rotation cultivar in 1943 (renamed 'Grasslands Manawa' in 1964) (Corkill, 1949). In 1950s and 1960s, further backcrossing of 'Grasslands Manawa' to PRG cultivars occurred, leading to the release of 'long-rotation hybrid' cultivar marketed as a perennial, 'Grasslands Ariki' (Barclay, 1963).

In the late 1960s, the 'Mangere ecotype' from the farm of Mr. Trevor Ellett in South Auckland was discovered (Corkill et al., 1981). The 'Mangere ecotype' was superior to the germplasm of 'Grasslands Ruanui' and 'Grasslands Ariki' in terms of erect leaves, larger tillers, higher winter production, greater resistance to summer drought, and rapid response to autumn rains (Bahmani et al., 2002; Corkill et al., 1981) and as well as high crown rust (*Puccinia coronata*) resistance and low stem rust (*Puccinia graminis*) occurrence (Stewart, 2006). Thus, the 'Mangere ecotype' provided a step-change in New Zealand's pasture improvement and was the mother material for many cultivars that have subsequently been bred in New Zealand (Easton et al., 2011). 'Grasslands Nui' was the first PRG cultivar derived from the 'Mangere ecotype' and development was completed by DSIR Grasslands Division in 1975 (Armstrong, 1977; Hayman, 1980). Subsequently, seeds of the 'Grasslands Nui' cultivar was first commercially available in 1976 (Armstrong, 1977).

During the period from 1973 to 1978, certified and commercially available PRG cultivars accounted for 70% of the total pasture sowings in New Zealand. 'Ruanui', 'Grasslands Nui', and 'Ariki' cultivars comprised approximately 70%, 20%, and 10%, respectively, of the total amount of retail sales of PRG cultivars in the late 1970's (Hayman, 1980; Lancashire et al., 1979). During that time, 'Grasslands Nui' developed a reputation as a superior PRG cultivar due to its high summer yield. For example, an experiment carried out to compare the summer performance of 'Grasslands Nui', 'Ariki' and 'Ruanui' (i.e. sown in combination with clover at different levels of irrigation under sheep grazing in Canterbury) showed that 'Grasslands Nui' was more persistent and higher yielding than the other two cultivars although the weight gain in sheep grazing it did not differ significantly

from that of other two cultivars (Armstrong, 1977). Similar results were also reported from subsequent trials (Hayman, 1980; Percival & Duder, 1983). A second cultivar from the Mangere ecotype, Ellett, was released in 1980 by a private seed company. Overall performance of Ellett was comparable with 'Grasslands Nui' and both cultivars yielded more than the reference cultivar 'Grasslands Ruanui' (Lancashire et al., 1979; Wratt & Smith, 1983).

Artificial doubling of the chromosome number using colchicine for bigger leaves was first achieved in *Lolium* species in 1930s in the USA (Myers, 1939) and was investigated in New Zealand starting from the late 1950s (Ahloowalia, 1967). As a result, the first tetraploid cultivar 'Grasslands Tama' was developed from an annual form of ryegrass (Ahloowalia, 1967) and released in 1968 (Armstrong, 1981).

Later, many other tetraploid ryegrass cultivars (of both perennial and hybrid ryegrass) were developed, including 'Grasslands Greenstone', 'Nevis', 'Quartet', 'Ceres Horizon', 'Grasslands Sterling', 'Bealey', and 'Banquet'. These tetraploid cultivars were characterised by increased tiller size (although offset by decreased sward tiller density), high palatability, and lower summer production and persistence than diploids when subject to the same grazing intensities (Ahloowalia, 1967). Hence, several comparative yield trials were undertaken to differentiate the yield advantage between tetraploid and diploid ryegrass cultivars, where the two-ploidy types were provided with differential grazing methods (i.e. adjusted grazing heights) in order to avoid possible confounding effects of grazing affecting performance through their major differences in leaf size and tiller numbers (Chapman et al., 2015). Tozer et al. (2014) found that older and tetraploid pasture cultivars had lower tiller density than that of diploid perennials. However, it was reported that low tiller density may reduce summer yield and persistence of tetraploids (Chapman et al., 2015). More recent opinion is that tetraploid cultivars require high soil fertility and good farm management to fully express their growth potential and provide improved animal intake and production (Lee et al., 2012).

In the 1980s, PRG germplasm was collected from regions of North West Spain which have an oceanic climate with summer rainfall and temperature similar to that in New Zealand and used in new cultivar development by New Zealand plant breeders (Stewart, 2006). It was anticipated that this germplasm would possess

better summer drought tolerance than that of the germplasm imported by settlers from the United Kingdom (Easton et al., 2011; Stewart, 2006)..

After Plant Variety Rights Legislation was enacted in 1987, private seed companies such as NZ Agriseeds Ltd., PGG Wrightson Seeds Ltd. and Cropmark Seeds Ltd. were involved in forage grass breeding and with the introgression of Spanish germplasm into the local material. The cultivars developed by introgression of Spanish germplasm into the New Zealand PRG breeding system are listed by Stewart (2006), and generally display strong winter growth activity, late flowering and a low vernalization requirement, and an excellent resistance to crown and stem rust (Stewart, 2006). An early example was 'Grasslands Impact' released in the 1990s and bred from a cross between introduced Spanish germplasm and 'Grasslands Nui'. Later, several cultivars including 'Tolosa' and 'Arrow' (diploids released by NZ Agriseeds Ltd.) and 'Banquet' (a tetraploid released by NZ Agriseeds Ltd.) were derived from 'Grasslands Impact' (Stewart, 2006). Also, national trials were initiated by New Zealand Plant Breeding and Research Association (NZPBRA; an association of commercial seed companies) to evaluate newly-bred PRG cultivars in 1991 (Easton et al., 2001). Results from national trials confirmed that new cultivars incorporating the Spanish germplasm yielded 6% and 9% more annually and seasonally (i.e. mainly in summer), respectively, than the older cultivars they had superseded (Easton et al., 2001).

In the 2000s, public and private plant breeding bodies in New Zealand have organised collaborative collection expeditions to search for a diverse drought-tolerant PRG genetic material from the Eastern Mediterranean region (Easton et al., 2011; Stewart, 2006). The aim of these expeditions was to capitalise on the new source of genetic variation through the introgression of Mediterranean germplasm into the local material when breeding PRG for persistence. However, Stewart (2006) asserted that the high genetic variation existed in the Mediterranean germplasm collected from expeditions was underutilised in the early 2000s and suggested its importance for future pasture improvement programs. Meanwhile on-going development of cultivars incorporating Spanish germplasm occurred, with two commercially successful examples being 'Trojan' (a re-selection of 'Tolosa' for better seed yield, released by New Zealand Agriseeds Ltd.) and 'Ceres One50' (a

crossed cultivar taking ‘Tolosa’ as one parent, and released by ‘Agricom’). In addition, the incorporation of endophytes into new cultivars was a continuous part of this breeding process since the 1990s (Lee et al., 2012) because the importance of fungal endophyte (formerly known as *Neotyphodium spp*., now renamed *Epichloë*) for both animal health and insect deterrence was recognised in the early 1980s (Section 2.1.4 above) (Fletcher & Harvey, 1981; Latch & Christensen, 1982).

Building on the various PRG improvement initiatives and considering future climate predictions in New Zealand reviewed above (Section 2.2.2), there may be an emerging industry need to improve drought tolerance of new PRG cultivars (Hutching & Moor, 2017; Johnston, 2013; Lee et al., 2012; Williams et al., 2007). In the recent past and currently, the primary emphasis has been placed on higher yield with characters such as drought tolerance assessed only indirectly through field observations at various stages in the cultivar development process (Casler et al., 1996; Easton et al., 2011; Lee et al., 2012; Rogers et al., 2019). However, PRG drought tolerance is to be achieved by a combination of morphological (i.e. tiller density, leaf elongation, leaf senescence, deep rootedness) and as well as physiological (i.e. LWP, RWC, OP, SC, Pn), and biochemical (i.e. WSC, proline, ABA) traits as noted in Section 2.2.4 above. The question on how to introduce such specific traits at the scale of a commercial breeding operation, when it is being done now through germplasm selection and field observation at the half-sib family level but not through trait selection at the genotype level, is not straight forward. As noted in Chapter 2.3.1 above, WUE is one such important physiological trait that has seldom been researched in PRG selection and breeding programs.

2.3.3 Plant breeding principles related to PRG drought tolerance trait evaluation and enhancement

2.3.3.1 Selection of parents

Breeding of cross-pollinated species generally focuses on population improvement rather than the improvement of individual plants (Acquaah, 2012). Breeders working with PRG, a cross-pollinated forage species (Thorogood & Hayward, 1991), typically produce so-called ‘synthetic’ cultivars that are populations of related, but

genetically different individuals derived from several parent plants that are chosen for target traits (Acquaah, 2012; Lee et al., 2012).

Pasture breeding programs, including those involving PRG, are generally initiated with the crossing of elite plants that are chosen from a genetically variable source population (e.g. germplasm accessions, or survivors from old pastures in the field) through phenotype screening for target traits either in the controlled environment or field settings (Acquaah, 2012; Casler, 1995; Casler et al., 1996; Casler & Brummer, 2008; Lee et al., 2012). Thus, one of the most critical decisions that a plant breeder should make is the selection of parental germplasm (i.e. source population) to satisfy set goals of the breeding project (Fehr, 1991). The breeder is required to assign priorities among all the traits of interest based on the economic importance of each trait and farmer's preference for the performance of the desired cultivar (Acquaah, 2012). Also, this selection of elite parents for the target traits involves the selection of an elite germplasm with the best mean performance for each trait and the greatest possible within-population genetic diversity (Bertan et al., 2007; Fehr, 1991). For that reason, it is important to use parents from various backgrounds to keep a high genetic diversity within the source population, without unreasonably sacrificing the good performance of potential traits (Acquaah, 2012; Bertan et al., 2007; Fehr, 1991).

Commercial PRG cultivars are considered to be one of the best sources of parental germplasm as the mean performance of such a population is generally high for characters of major importance (i.e. yield) (Fehr, 1991). Elite breeding lines, particularly those at the advanced stages of testing, are also suitable as parents because pasture breeders usually work with superior lines in breeding populations to increase the potential yield gain in every year (Fehr, 1991).

2.3.3.2 Selection of progenies: Phenotypic & family-based selection

Progeny testing and recurrent selection by means of growing half-sib (HS) families from open pollination and poly-cross programs is the most commonly used strategy in PRG breeding programs in temperate countries (Aastveit & Aastveit, 1990; Wilkins, 1991).

Recurrent selection improves the mean performance and maintains the genetic variability of a population for quantitatively inherited traits, with enhanced frequency of desired alleles cumulative over several cycles of selection (Acquaah, 2012; Brim & Burton, 1979; Fehr, 1991; Ravel & Charmet, 1996). Also, recurrent selection provides multiple opportunities to advance a given population through intermating of genotypes within that population, with high flexibility to add new germplasm in to the source population whenever the genetic base of the population gets narrower as a result of repetitive selection (Acquaah, 2012; Hallauer et al., 2010; Hull, 1945; Ravel & Charmet, 1996). In the early stages of the breeding process, pasture selection experiments are generally restricted to a manageable size and conducted as representative trials (i.e. glasshouse pot trials, rainout shelter experiments, or small field trials) (Lee et al., 2012). As the breeding line advances, an extensive evaluation is to be done in large field trials with many replications and several sites for a period of 10–15 years before releasing a new synthetic cultivar (Acquaah, 2012; Lee et al., 2012).

The most common recurrent selection method is the phenotypic or mass selection that refers to the selection of individual plants based on the phenotypic performance (Acquaah, 2012). Genotypes selected based on this method are characterised by both genetic variance components and environmental variance within and across populations and trial sites, that generally masks the true genotypic value and lowers the efficiency of the selection or extends the length of a single selection cycle (Hallauer et al., 2010; Posselt, 2010). Thus, phenotypic selection methods are appropriate only for traits with high narrow-sense heritabilities or high additive genetic variance (i.e. low non-additive genetic variance and genotype × environment interaction effect) to achieve high genetic gain at high selection intensity within short breeding cycles (Acquaah, 2012; George, 2014; Posselt, 2010).

For the selection of PRG breeding populations, parental phenotype has been at times envisaged in progeny performance in breeding programs. Also, visual ratings and scores for vigour, tiller number, leaf elongation, and shoot dry matter of parent plants were often used as proxies for the average performance of yield in PRG field trials under summer drought events (Casler & Brummer, 2008; Lee et al., 2012). However, Casler & Brummer (2008) reported that the genetic correlation between such traits and herbage yield was less significant. This may be due to the lack of progeny testing or the ignorance of heritability estimates of common selection traits

or incomplete quantitative genetic information available for such traits. For similar reasons, there has been a yield lag in forage species relative to that of grain crops for many years (Casler, 1997; Casler et al., 1996; Casler & Brummer, 2008). It was suggested that family-based selection methods may reduce inaccuracies of the aforesaid method (Casler & Brummer, 2008; Wilkins & Humphreys, 2003).

Family based selection methods were introduced for traits with low heritability and high G×E effects particularly, for situations where phenotypic selection methods become erratic and unreliable (Acquaah, 2012; Posselt, 2010). In this method, combining ability or productivity in crosses (defined as the elite parents' ability to combine among each other during crossing such that desirable genes or characters are transmitted to their progenies), is basically tested. For cross-pollinated forage species like PRG, general combining ability of parental clones (i.e. GCA; the average performance of the progeny of one genotype in a series of crosses) is widely evaluated through the half-sib progeny test procedure to develop synthetic cultivars, to recombine selected entries in recurrent selection programs, and to obtain quantitative genetic information (Aastveit & Aastveit, 1990; Nguyen & Sleper, 1983). The basic feature of the HS selection method is that HS families are prepared for both evaluation and recombination within one generation (Acquaah, 2012). Thus, populations are created by random open pollination of selected female plants and seeds from the families generated in the first generation and evaluated in replicated trials in different environments (Acquaah, 2012). As noted in Section 2.1.2.2, the naturally-developed self-incompatibility system exists in most PRG genotypes, and prevents self-pollination to a large extent.

Through conducting specific mating designs, the genetic influences of a genotype can be partitioned into additive and non-additive components. With the poly-cross design or HS family selection approach, the total additive variance is halved because the female parent functions as the tester in the selection procedure (Acquaah, 2012). The HSF mating system also allows proper partitioning of additive genetic variance among- and within- HSFs and thus, the collective approach (among and within family selection; AWFS) extracts the total additive genetic variance of target traits in the breeding population (Acquaah, 2012; Casler & Brummer, 2008; Fehr, 1991). Theoretically, the covariance among non-inbred half-sib families is ¼ of the total

additive variation (σ^2_A) (Casler & Brummer, 2008; Hallauer et al., 2010). Thus, the expected genetic gain (ΔG_c) from among family selection is calculated as; ΔG_c = (¼iσ^2_A)/ σ^2_P , where 'σ^2_P' is the phenotypic variance among half-sib families and 'σ^2_A' is the total additive genetic variance among half-sib families, and 'i' refers to the selection intensity (Casler & Brummer, 2008). Within-family selection of superior half-sib families exploits the remaining ¾ of the total additive genetic variation (Casler & Brummer, 2008). Nevertheless, the AWFS is unable to distinguish between genotypic and environmental variation, unless each progeny of each family is clonally replicated (Aastveit & Aastveit, 1990).

2.3.3.3 Estimation of genetic parameters of a breeding population

2.3.3.3.1 Population variation: phenotypic and genetic variance estimates

Population diversity is generally estimated as the genotypic variation in a given population for the phenotypic variance of measured traits where the phenotypic expression of a plant trait is determined by both the genetic and environment components (Fehr, 1991; Nyquist & Baker, 1991). The phenotypic variance of a given trait represents the collective effect of its (i) genetic variance (ii) experimental error or environmental variance (i.e. a measure of differences among phenotypes caused by the failure to treat each genotype exactly alike) and or, (iii) genotype × environment interaction (i.e. the failure of genotypes to perform similarly to each other when they are evaluated in different locations and/or years and comprising the sum of genotype × location, genotype × year, and, genotype × location × year interaction effects) (Fehr, 1991). Therefore, genetic variance, that is to be exploited through selection for the genetic improvement of target traits, is often masked by environmental stimuli (Fehr, 1991).

For a quantitative trait of an individual plant, the diversity in gene frequencies of a population, which is termed genetic variance, influences the total gene action and determines the differences between individuals within a population or between populations (Acquaah, 2012; Falconer, 1960). The estimate of genetic variance components include additive, dominance, and epistatic variances that are important for characterizing a given population or populations in conjunction with the population mean and also for calculating quantitative genetic parameters of a given

trait or traits (e.g. heritability, genetic gain, and selection differential) (Acquaah, 2012). For cross-pollinated pasture species, the additive gene performance of HS families is important (Nguyen & Sleper, 1983). Additive genetic variance of HS families is generally computed as an estimate of GCA of parents (or the covariance between the parents and HS families) or by averaging additive genetic variances of all the poly-crosses (i.e. analysis of variance between and within HS families) while keeping one parent in common in a given poly-cross mating system (Acquaah, 2012; Nguyen & Sleper, 1983; Nyquist & Baker, 1991).

2.3.3.3.2 Heritability

Heritability is a measure of the relative importance of heredity or the ratio between the genotypic variance (σ_G^2) and the phenotypic variance (σ_P^2) of a trait. Heritability is further specified as having two categories: (1) broad-sense heritability (i.e. h_b^2; the ratio between the total σ_G^2 and the σ_P^2) and (2) narrow-sense heritability (i.e. h_n^2; the ratio between additive genetic variance or σ_A^2 and the σ_P^2) (Acquaah, 2012; Falconer, 1960; Nguyen & Sleper, 1983; Nyquist & Baker, 1991).

The heritability of a trait has a major impact on the method of selection in breeding programs as it determines the relative effectiveness of several traits for selection and the transferability of the target traits through generations (i.e. mass selection is applicable for highly heritable traits) (Fehr, 1991). Thus, the direct selection method is employed for highly heritable traits. The indirect selection is recommended whenever the heritability of the primary trait is lower than that of the secondary trait, particularly when there is a high genetic correlation between those two traits (Acquaah, 2012; Fehr, 1991).

According to Fehr (1991), h_n^2 is the most important type of heritability as it measures the relative importance of the additive proportion of the genetic variance. Narrow-sense heritability is directly transferred from parents to the progeny and is indicative of the predicted genetic gain per selection cycle (Acquaah, 2012; Fehr, 1991). Hill (1977) asserted that h_n^2 is more important than h_b^2 in forage grass breeding because non-additive genetic variances are generally negligible for common breeding methods utilized in cross-pollinated species (e.g. a poly-cross that exploits GCA).

The heritability of a particular trait is not a constant value throughout the cyclic selection process (Nyquist & Baker, 1991). Therefore, a better understanding of genetic factors that contribute to the heritability of traits, may permit the breeder to develop an efficient breeding program with the use of minimum available resources (Fehr, 1991; Nyquist & Baker, 1991). For example, the linkage disequilibrium that occurs when the frequency of coupling and repulsion phase linkages are not equal at every crossing phase of each selection cycle may influence the heritability estimates. Linkage disequilibrium may cause either an upward (increase) or downward (decrease) bias to the estimates of additive and dominance genetic variances, and consequently to the heritability estimates of a given trait (Fehr, 1991; Jia, 2017). Fehr (1991) and Nguyen & Sleper (1983) suggested that the linkage disequilibrium can be eliminated by random-mating prior to estimating the heritability of traits in a given population. That may maintain the highest possible genetic variance within the source population with a less chance of dominance and epistasis or inbreeding, allowing adequate randomization in the sampling process in each selection cycle (Fehr, 1991).

2.3.3.3.3 Genetic gain

Genetic gain (ΔG_c) of a breeding program is the change in the mean performance of a population from one selection cycle to the next (Acquaah, 2012; Fehr, 1991). Therefore, ΔG_c provides information about the relative efficiencies of different plant breeding methodologies (Acquaah, 2012; Bertan et al., 2007; Fehr, 1991; Nyquist & Baker, 1991). The magnitude of the genetic gain of any plant species depends on several factors such as the heritability of the traits of interest, biology of the species, duration of the breeding cycle, arrangement of spaced-plants in field trails, and the availability of resources in any plant breeding method (Fehr, 1991).

The standard equation that interprets the ΔG_c of a selection trait per breeding cycle, is often presented as the breeders' equation: ΔG_c (also referred as 'R' in literature) = h^2D, where 'h^2' is the narrow-sense heritability and 'D' is the selection differential (Fehr, 1991). The selection differential of traits of interest is estimated using the difference between phenotypic means of the parent and progeny populations of each selection cycle. For example, if the population mean is 100, and a subpopulation is selected with a mean value of 125 to breed the next generation, and the heritability is

0.50, then ΔG_c equals to 0.50 × (125 – 100) = 12.5. This basic equation may be modified using the standardized selection differential or the same selection intensity over cycles and by increasing the proportion of σ_A^2 in the test population through an increased parental control (i.e. selection of parents with high combining ability for desired traits) or altered family structure (Bernardo, 2002). This can also be achieved by reducing non-genetic effects or the environmental variance, for a better definition of the relative efficiency of a given breeding method (Bernardo, 2002, 2010).

Genetic gain is defined in two ways: (1) realized ΔG_c and (2) predicted ΔG_c (Rutkoski, 2019). The realized ΔG_c was described as the change in the average breeding value of a population over cycles of selection for traits of interest. The predicted ΔG_c was evaluated using a modified version of the standard equation of ΔG_c for one single selection cycle;

$$\Delta G_c = k_f c \frac{\frac{1}{4}\sigma_A^2}{\sigma_{PF}}$$

Where, ΔG_c is predicted genetic gain using among HS family selection, σ_A^2 is additive variance, σ_{PF} is among family phenotypic standard deviation, k_f is among family selection intensity and c is parental control (0.5).

Previous forage selection experiments have compared the relative importance and practicality of both types of ΔG_c (Casler, 1997; Hopkins et al., 1993; Mitchell et al., 2005; Resende et al., 2013). The consensus is that the realized ΔG_c is more useful to the breeder than the predicted ΔG_c. However, it is difficult to establish 'realized ΔG_c' precisely as it is difficult to maintain the consistency of genotype behavior in different drought challenge events (in multiple drought cycles) in selection experiments (Rutkoski, 2019). For example, additive genetic variances were overestimated due to G×E effect biases in field settings (Fè et al., 2015; Rutkoski, 2019). Therefore, predicted ΔG_c was identified as a precise representation of realized ΔG_c and also a practically-feasible approach to evaluate the expected level of ΔG_c of measured traits in quantitative genetic analyses (Rutkoski, 2019).

2.3.3.3.4 G×E effect

Genotypes in a given population do not exhibit the same performance relative to each other across different environmental conditions (Fehr, 1991). Changes in the individual performance across environments limit the effectiveness of the selection and accordingly, modify the ΔG_c of superior genotypes in each season or year (i.e. mostly a downward progress) (Fehr, 1991; Hill, 1975). Thus, quantification of the G×E effect that describes individual responses of genotypes in a population to a given environmental condition becomes important for plant breeding purposes (Acquaah, 2012).

Pasture fields are generally exposed to a wide range of environmental variables such as different soil types, soil moisture and fertility levels, temperatures, light or shade levels and cultural practices (Acquaah, 2012; Fehr, 1991; Hill, 1975; Nyquist & Baker, 1991). Therefore, ΔG_c of elite cultivars bred for a specific purpose should be defined excluding the environmental variance or experimental error factors (i.e. different row spacings, plots to plot variations, soil types, drainage patterns, planting or flowering dates, and plant population characters) in field experiments.

The adverse impact of the G×E effect on the selection efficiency can be eliminated by knowing its magnitude and that is generally achieved by evaluating breeding lines in multiple environments and years (or selection cycles) (Acquaah, 2012; Fehr, 1991; Hill, 1975; Nguyen & Sleper, 1983). For this reason, plant breeders gather information on the relative importance of genotype × location, genotype × year, and genotype × location × year interaction effects to have a clear idea about the magnitude of the environment component in field trails (Fehr, 1991). Thus, improved cultivars are often tested in different geographical areas to determine whether a single cultivar selected for a specific character (i.e. moisture deficit tolerance) under prevailing environmental conditions in one location can exclusively be recommended for several other areas with similar environmental conditions (Fehr, 1991). Consequently information on the stability of the performance of superior genotypes across different environments may help the breeder to recommend them for a wide range of growing conditions that are likely to occur in farmers' fields (Des Marais et al., 2013; Mahon, 1983; Pauli et al., 2016; Ray & Harms, 1994). However, glasshouse experiments have been suggested as ideal for

estimating quantitative genetic parameters of target traits because such experiments generally have low G×E effects, meaning that ΔG_c will be higher than that is estimated in field settings (de Almeida Silva et al., 2012; Nguyen & Sleper, 1983). It has been suggested that high positive phenotypic correlations between traits of interest may shorten selection cycles, if the environmental variance is small (Acquaah, 2012; Dudley & Moll, 1969; Fehr, 1991; Gardner, 1963; Nguyen & Sleper, 1983).

2.4 Implications of quantitative genetics and multivariate statistical analyses in selecting and breeding PRG for drought tolerance trait response patterns

Historical progress in crop breeding through conventional direct selection (i.e. selecting genotypes for high yields under drought stress) has been slow due to large G×E effects exist in field settings (Hill, 1975; Richards et al., 2002). However, well-managed field studies showed that direct selection of grain crops for yield can be effective, if yield and associated traits exhibit high h^2 (Atlin et al., 2004; Kumar et al., 2008). Also, most selection experiments have emphasized the importance of quantifying heritability estimates of drought tolerance traits of grain crops (Condon & Richards, 1992; Dudley & Moll, 1969; Mathew et al., 2018; Rebetzke et al., 2001; Srivastava et al., 2017) and PRG (Lee et al., 2012; Robins & Alan, 2016; Thomas, 1990) for breeding purposes. Therefore, certain drought tolerance traits have been more frequently considered in quantitative genetic analyses. For example, heritability estimates OP under drought were documented in relation to wheat (Mathew et al., 2018; Moinuddin et al., 2005), sorghum (Basnayake et al., 1995), rice (Atlin et al., 2004; Srivastava et al., 2017), creeping bentgrass (Lehman & Engelke, 1991) and, PRG (Thomas, 1990). John et al. (2011) and de Almeida Silva et al. (2012) suggested that species-specific studies that discuss quantitative genetic information of the WUE trait in pre-breeding experiments are of utmost importance, if drought tolerance is set as a major breeding objective. Accordingly, high estimates of heritability were found for WUE and associated traits in some herbaceous plants like *Lobelia siphilitica* suggesting that WUE may be a beneficial drought tolerance trait for the studied species (Caruso et al., 2005). Moreover, a study conducted using three cool-season grass cultivars (crested wheatgrass, western wheatgrass, intermediate wheatgrass, and reed canary grass) reported moderate to high

heritability estimates (i.e. averaging from 69% to 94%) for WUE in both drought and well-watered conditions and suggested that the WUE trait is a potential selection criterion for drought tolerance in forage species (Barker et al., 1989). However, a complete picture of the quantitative genetic information of WUE was lacking for studied species (de Almeida Silva et al., 2012; Easlon et al., 2014; Feldman et al., 2018; Lopez et al., 2017) and also, such information has seldom been documented in conjunction with water relations trait response patterns of PRG.

Incomplete quantitative genetic information on the WUE trait may be partly due to the fact that the importance of WUE has always been overlooked as a selection criterion for plant drought tolerance (Section 2.2.4.1) (Blum, 2005; Blum, 2009). Actual WUE has long been considered to be a labour intensive trait measurement due to its low throughput nature in both controlled glasshouse (Feldman et al., 2018) and field environments (Moghaddam et al., 2013). Nevertheless, results of a series of glasshouse studies conducted by a former Massey PhD student suggested that one prospect for future PRG research may be the selection of elite genotypes displaying high WUE within New Zealand PRG germplasm (Figure 7.2 in (Hussain, 2013). Also, recent experiments suggested the importance of WUE as a drought tolerance trait in different species (Section 2.2.4.1). However, most such suggestions have been based on phenotypic observations of WUE trait behaviour. Therefore, it is worth estimating quantitative genetic information of WUE in a controlled environment to realize the relative degree of genetic control over the phenotypic performance of this trait. Such information may convince breeders to consider this trait for pasture breeding purposes in future.

As described in Section 2.2.4.1 above, WUE can be quantified in two different ways: (i) the whole plant-level WUE or agronomic WUE (i.e. plant water consumption per unit of plant dry matter production; g WU/g DM or yield; WUE) (Blum, 2009; de Almeida Silva et al., 2012; Ullaha et al., 2019) (ii) intrinsic or instantaneous water-use efficiency at the individual leaf level (i.e. the ratio between photosynthesis and transpiration; WUEi or WUE_{AE}) (Condon et al., 2002; Rebetzke et al., 2002; Richards et al., 2002) or more descriptively as daily integrals of leaf transpiration efficiency (Medrano et al., 2015). Due to the fact that conventional glasshouse experiments performed by pot-weighing studies to estimate WUE were

laborious, time-consuming and not necessarily representative of field conditions (Moghaddam et al., 2013), WUEi has largely been utilized in drought tolerance definitions in most studied crops (Chen et al., 2011). In particular, carbon ($\Delta^{13}C$) or oxygen ($\Delta^{18}O$) isotope discrimination has often been adopted as the proxy and more easily measurable trait for WUE and as an indicator for the overall drought performance in studied crops (Adiredjo et al., 2014; Akhter et al., 2010; Ehdaie et al., 1991) including cool season grass species (Ebdon & Kopp, 2004; Ghannoum et al., 2002). For the same reason, the large majority of literature has discussed quantitative genetic parameters of WUEi or $\Delta^{13}C$ representing WUE in a variety of crops including forage species (Barker et al., 1989; Chen et al., 2011; Condon, 2020; Condon & Richards, 1992; Condon et al., 2002, 2004; Gresset et al., 2014; Inostroza et al., 2015; Johnson et al., 1990; Johnson & Bassett, 1991; Ray et al., 1999). Hence, the direct measurement of WUE was underutilised for breeding purposes (Donovan & Ehleringer, 1994; Easlon et al., 2014; Ehdaie et al., 1991; Feldman et al., 2018; Johnson & Asay, 1993). However, documented evidence on the reliability of quantitative genetic estimates for WUE proxies when desiring to understand the quantitative genetic behaviour of WUE is almost absent from literature describing pasture cultivar selection programs targeting drought tolerance.

It has long been understood that drought tolerance, as a combined performance of a number of plant drought responses that are expressed differently and concomitantly, should be addressed by selecting plants for a beneficial trait combination (Blum, 2005). Therefore, a good understanding of the patterns of physiological and morphological trait data distribution, that are related to the improved WUE and high yield under stress may allow the breeder to establish efficient pasture selection methods (Farshadfar et al., 2001; Richards et al., 2002). The literature suggests that there are three inter-dependant trait associations that can potentially be exploited in breeding crops for high WUE: (i) efficient soil-moisture uptake with the involvement of deep rootedness, (ii) leaf OA for improved leaf conductance and assimilation, (iii) balanced assimilate partitioning between shoots and roots through plant's biochemical and physiological adaptations in drought. (Condon et al., 2004) asserted that the progress in wheat breeding for drought tolerance may be achieved by pyramiding traits related to (i), (ii), and (iii) above. Accordingly, the question becomes which one or which ones among the drought tolerance trait responses

known from other species is drought-response physiology of PRG characterised by? This then suggests that scientists seek relevant data as evidence.

Principal component (PC) analysis is one of the versatile statistical analyses that is capable of providing an overview of complex multivariate trait data (Bro & Smilde, 2014) through the reduction of the dimensionality and identification of directions (i.e. PCs) of the data set while retaining most of its variation (Abdi & Williams, 2010). Accordingly, the first PC is the direction along which the samples show the largest variation. The second PC is the direction uncorrelated to the first PC and shows the second largest variation. The same declining pattern of data distribution variation is seen in other PCs as a sequence (Ringnér, 2008). As PCA generates different uncorrelated groups (PCs) of inter-correlated quantitative dependent variables, this method can be used for revealing trait associations that are more complex than the simple correlations between the traits. Most commonly, results from PC analysis are presented to highlight dominant data distribution patterns as extracted in the form of a complementary set of score and loading plots for the first two PCs (Chapman et al., 2002; Jolliffe, 2003; Ringnér, 2008). However, there is growing recognition that lower-order PCs can at times isolate highly interesting biological signal or information on outliers of experimental results (Jolliffe & Cadima, 2016; Khaembah et al., 2013; Sartie et al., 2018; Sartie et al., 2011). Accordingly, there is growing use of PCA for this purpose in multi-trait agronomic investigations, in order to understand complex trait associations that are either demonstrated by a subset of plants within a population or are describing physiological processes otherwise masked by larger effects such as data variation arising from plant age or plant size (Griffiths et al., 2017; Sartie et al., 2009). This latter approach is often adopted in this thesis.

2.8 Conclusions

With New Zealand's heavy economic dependence on pastoral industries, the additional pressure exerted by ever-increasing summer droughts on the persistence and the overall productivity of pastoral agriculture has become a major challenge to pasture breeders.

Industry breeding objectives for pasture improvement have evolved with time and particularly, reoriented towards the enhancement of summer performance in the recent past. However, the progress made by PRG improvement for drought tolerance has been slow due to (1) inadequate knowledge on complex drought response patterns, (2) the use of conventional and inefficient selection strategies, and (3) inconclusive genetic information available for key selection traits in pasture breeding programs.

The advantage of selecting plants for traits targeting pasture survival and production maintenance (i.e. WUE) has been disputed in previous literature. However, research evidence to embrace such information for PRG breeding purposes is lacking in forage research. As an alternative, a range of acknowledged drought-response trait measurements that are associated with efficient WU are most likely to allow the pasture breeder to utilize WUE as a potential selection criterion in breeding PRG for drought tolerance. As a cross-pollinated species, a population of related, but genetically different individual, PRG genotypes would be expected to provide adequate scope for population improvement for WUE. However, phenotypic differences observed between genotypes for the trait measurements are a result of complex G×E effects that are most likely to mask the true genetic effect of trait measurements under drought.

Despite New Zealand's extensive breeding effort over the years, there has been comparatively little research on the within-population variation that exists for WUE under drought events. It is clear that there is a gap in the pasture breeding research, and a need for research that explores the importance of WUE as a key selection trait for PRG drought tolerance and beneficial associations between WUE and key plant water relations traits. Considering the importance of the constructive associations between drought-response traits related to efficient WU of PRG and the complexity of such trait responses where G×E effects occur, it is also advisable to investigate the genetic background of a range of drought-response patterns for pasture breeding purposes under controlled environment conditions.

Chapter 3

Preliminary screening and divergent selection of a range of New Zealand PRG germplasm for WUE

3.1 Abstract

This chapter describes the first experiment of a series of four glasshouse experiments which screened elite PRG germplasm possessing high WUE and favourable water relations traits from populations of three commercially available PRG cultivars representing a time series of release dates (220 genotypes: Grasslands Nui, Nui, 50 genotypes; Grasslands Samson, Samson, 80 genotypes; Trojan, 90 genotypes; 2 replicate clones of each genotype) under imposed drought. At three months of age and after root system development with pot water status near field capacity (FC), plants were grown on for a month under strong drought (at 60% FC) and then morphological characters, plant water relations traits and gas exchange measurements were performed to evaluate key drought-response patterns. The comparison of the 220 individual PRG genotypes confirmed large within-population variation for WUE (ranging from <100 to >1000 g WU/g DM) and most measured traits together with significant cultivar effects ($p < 0.05$) and a good agreement between clonal replicates for the key traits. Based on results of principal component (PC) analysis, a group of PRG genotypes was identified with a key drought-response trait association (i.e. PC3 from the PCA, explaining 13% of the total population variation) characterised by high SDW, improved WUE, more negative osmotic potential (OP), higher root dry weight at depth (RDW_D), high post-cutting regrowth, and slower soil moisture (SMC) drawdown. Accordingly, two contrasting groups of PRG genotypes were selected from the three cultivars (twenty and fifteen low- (HWUE) and high-WU (LWUE) genotypes, respectively). In this divergent selection, the HWUE selection displayed approximately a two-fold improvement in WUE together with improved SDW, RDW_D, and tissue hydration compared to those of the LWUE selection. Notably, the cultivar differences observed for the key traits were more pronounced in Trojan than in the other cultivars, suggesting that commercial PRG breeding has indirectly resulted in high WUE and drought tolerance in modern cultivars.

3.2 Introduction

With New Zealand's heavy economic dependence on pastoral industries (see Section 2.1.3 above), recent industry research effort has been directed at PRG improvement for summer yield (Cyriac et al., 2018; Lee et al., 2012). Perennial ryegrass has an acknowledged poor tolerance of summer drought and that has been the major cause for the seasonal fluctuations in the productivity of pastoral agriculture under the consequences of varying climate as noted in Section 2.2.2 above). Although improved summer performance has been addressed indirectly by the use of Spanish germplasm in PRG breeding programmes in New Zealand since the early 2000s (Stewart, 2006), the possibility of selecting PRG directly for productivity traits related to the efficient WU under water deficit has received little attention in pasture breeding programs in New Zealand. Despite the application of numerous selection methods for screening PRG for summer performance, there has been comparatively little research on the within-population variation that exists for WUE in commercially available cultivars.

Water-use efficiency (g DM/g WU (Blum, 2005) or g WU/g DM) or effective use of water (Blum, 2009) is a an underutilized but potential selection trait, particularly when the breeder's target is to achieve high average yields under moisture deficit conditions (de Almeida Silva et al., 2012; Easlon et al., 2014; Passioura, 2012). As noted in Chapter 2.2.4.1, Blum (2009) advised against the selection of crops for high WUE under drought, arguing that fast-growing plant genotypes (i.e. selection for higher g DM/g WU) may exhaust soil water reserves and low WU genotypes (i.e. selection for lower WU/g DM) may result in a yield reduction due to a range of physiological responses that are generally induced under moisture deficit. Moreover, there is no single drought-response trait that is highly correlated with high yield and low WU of plants under imposed drought (Blum, 2005; Ludlow & Muchow, 1990; Tuberosa, 2012). Therefore, there is a need for research that identifies low WU plants (i.e. high WUE) with trait response patterns (i.e. plant ecophysiology responses) that reduce evapotranspiration demand without an adverse impact on the plant size and the available soil moisture.

Some plant traits interact synergistically to promote efficient plant WU that is supportive of plant production and survival under drought conditions (e.g. OP and

deep rootedness) (Blum, 2017; Cyriac et al., 2018; DaCosta & Huang, 2006; Jones & Turner, 1978; Morgan & Condon, 1986; Teulat et al., 1997). Blum (2005) argued that selecting plants for such physiological traits may cause a genetic shift towards dehydration-avoidant plant types and that is in contrast to a high-yield-potential phenotype. Blum (2005) further explained that dehydration avoidance is typically defined by plant survival strategies with or without the involvement of productivity traits. In addition, the literature suggests that summer dormancy strategies can also be involved with constructive drought survival strategies in PRG (Volaire & Norton, 2006; Volaire et al., 2009; Volaire et al., 1998). However, the summer dormancy strategy of Mediterranean and North African PRG germplasm was regarded as an unfavourable drought response for improving drought tolerance of PRG cultivars bred for use in New Zealand, because of the typical yield reduction associated with summer dormancy (Hussain, 2013; Matthew et al., 2012). For instance, historic breeding efforts taken to develop Mediterranean PRG varieties for local applications were unsuccessful at the commercial level in South Australia (Silsbury, 1961) and New Zealand (Vartha, 1975).

In the process of evaluating the drought tolerance strategies of PRG varieties from different genetic backgrounds, Hussain (2013) found that elite genotypes within the New Zealand-bred cultivar Grasslands Samson showed high DM production with low soil moisture depletion under drought. In this experiment, it was hypothesised that aforementioned drought response is a promising avenue for pursuing the development of PRG genotypes with high WUE in future drought research (1st hypothesis). Also, He (2016) examined plant water relations traits of seven market-leading PRG cultivars under imposed drought in a rainout shelter experiment and found that more negative OP was an important contributing trait to PRG summer drought tolerance. Similar findings had also been obtained from a pilot study conducted in summer of 2014/2015 using 27 plants each, of cultivars ‘Grasslands Nui’ (the reference cultivar), ‘Request’ (an advanced local cultivar) and Trojan (a cultivar bred by the introgression of Spanish PRG germplasm to the local material) (see 1A.1). Therefore, it was hypothesised that PRG summer performance is a result of a range of trait responses triggered under imposed drought (2nd hypothesis). Thus, data generated from the pilot study mentioned above were subjected to a PCA to explore drought tolerance strategies of test plants. Results indicated multiple plant

strategies for responding to moisture deficit, specifically (i) faster growth rate with strong soil moisture depletion but an associated high WUE (low g WU/g DW), (ii) small genotypes with low WU (consistent with the profile of a high-WUE plant predicted by (Blum, 2009)), (iii) hydrated genotypes with low WU that was apparently linked to stomatal adjustment, and (iv) hydrated large genotypes with high WUE, slower soil moisture depletion during drought, and more negative leaf OP at low LWP indicative of OA (see 1A.1). Water-use efficiency trait responses (iii) and (iv) above seemed to be beneficial for pasture drought tolerance as noted in Section 2.2.4. These findings also suggested that the selection of PRG for WUE may improve drought tolerance of New Zealand PRG cultivars, if it could be achieved in large populations in a commercial context.

Given the limited plant numbers of the pilot study, it was decided as a first step, with a larger but a practically feasible number of plants, to test the repeatability of the pilot study results, and to obtain a larger selection of elite plants for potential use in a prospective plant breeding project. Thus, the main objective of Experiment 1 was to identify key water relations traits that can be employed to screen a given PRG population for elite genotypes exhibiting improved WUE and improved summer performance. It was hypothesised that PRG, as a population of related but genetically different individuals (Thorogood, 2003), may possess adequate variability for within- and among- population selection (Cui et al., 2015) (3^{rd} hypothesis).

This chapter provides details including methodology, results and discussion and, conclusions of Experiment 1 that was designed based on 1^{st}, 2^{nd}, and 3^{rd} hypotheses above and conducted from November 2017 to February 2018. Accordingly, the results and discussion section comprises 3 subsections; Section 1 describes the population diversity of PRG for WUE and associated traits, Section 2 explores drought-response patterns and trait associations of the tested PRG germplasm, and Section 3 examines differences in the trait behaviour of divergently selected elite PRG genotypes and their cultivar effects for the key traits.

3.3 Materials and methods

3.3.1 Plant material

A “time series” of three high-yielding New Zealand PRG cultivars with an industry reputation for persistence was identified to form the source population for the experiment. These were ‘Grasslands Nui’ (Nui; a cultivar released in the 1970s and developed from the so-called ‘Mangere’ ecotype), ‘Grasslands Samson’ (Samson; a 1990s successor to Nui), and Trojan (a more recently released PRG cultivar which incorporated germplasm from Spain) (Stewart, 2006). Fifty, 80 and 90 PRG genotypes of Nui, Samson, and Trojan, respectively, carrying the AR1 endophyte, were used as the test plant material in Experiment 1. The rationale for these numbers was that 50 plants of a cultivar establishes a reliable population mean for traits of interest (Sartie et al., 2009). The pilot experiment had shown a higher mean yield and improved WUE for ‘Request’ (i.e. similar to Samson) and Trojan, compared to Nui, so the chances of finding elite plants with higher WUE were expected to be enhanced by weighting the numbers towards the more recently released cultivars.

3.3.2 Experimental set up

This study was conducted as a pot experiment in a glasshouse environment at the Plant Growth Unit, Massey University, New Zealand (40.3709° S, 175.6303° E, 35 m.a.s.l.) during summer, from June 2017 to January 2018.

The seeds were germinated in root trainers, and seedlings grown for 8 weeks to a size of ≥10 tillers to facilitate division of plants into two clonal replicates (4–5 tillers each). Pots (sections of PVC water pipe 50 cm tall and 10 cm in diameter, with an end cap made from a double thickness of nylon horticultural shade mesh) were lined with tubular polythene plastic for easy root harvesting at the end of the experiment (with perforations at the bottom to allow drainage and aeration) and a saucer kept at the bottom of each pot to capture excess drainage if there was any. Pots were filled with fertilised (A combined mixture of the recommended dosage of three fertilizers; the ratio of Lebonon Turf Woodace Long-term Fertilizer Mix to Dalton’s Short-term Fertilizer Mix to Dolamite was 2 to 1 to 11/2) ‘A’ horizon of an ‘Egmont Black Loam’ soil (a Typic Distrandept under USDA nomenclature, (Perrott & Sarathchandra, 1987)) with water holding capacity (WHC) >65%, measured as the mass of gravimetric soil water/mass of soil or MW/MS). Pots were packed to a

standard weight of 3.7–3.9 kg of air-dried soil per pot (MW/MS approximately 5% and bulk density 1.1–1.3 g/cm^{3}in each pot). Two clonal replicates of the 220 genotypes from three cultivars to be tested were transplanted into experimental pots and arranged in a randomized complete block (row-column) design in the glasshouse (Figure 3.1).

Figure 3.1 General view of the pot arrangement in Experiment 1: Two clonal replicates of 220 perennial ryegrass genotypes: Nui (50 genotypes), Samson (80 genotypes), Trojan (90 genotypes), with border plants; Abbreviations: Rep, replicate; R_1…R_n, Rows; C_1….C_n, Columns; E, Randomised experimental plants from three cultivars; B, Border plants (Note: The arrow in the figure indicates the weather data collection unit located at the canopy level inside the glasshouse; Photograph was taken when plants were growing with soil water content near field capacity and before imposing the water deficit treatment).

Pots were maintained at near field capacity (85–95% FC) for eight weeks to allow tillering to ≥20 tillers/pot and to ensure good root development. Genotypes were then defoliated to 5 cm above the soil surface and the drought treatment was imposed during the subsequent ‘measurement’ regrowth.

3.3.3 Drought treatment

The average daily maximum and minimum temperatures inside the glasshouse were 26 and 16°C, respectively. The relative humidity ranged from 60–80% and light intensity from 800–1000 μmol/m^2/s at the canopy level at midday. Pots were watered with measured amounts of tap water using a measuring cup by hand, depending on the soil moisture availability as determined by pot weight (i.e. by weighing pots individually on an electronic balance to 1 g precision) each time. Moisture deficit was imposed from mid-December 2017, starting with pots at field capacity (FC) and maintained at 85–95% FC for a month and then, increasing irrigation interval and reducing irrigation volume progressively until pots reached 60% of FC (MW/MS ~ 35%). Pots were then maintained at this soil moisture content for a one-month 'drought' period. Soil moisture availability at the key measurement period was estimated based on the experience from previous similar experiments for which a benchmark of predawn leaf water potential of randomly selected plants was considered (i.e. approximately –0.9 MPa) to facilitate an adequate physiological adjustment of PRG genotypes tested under imposed soil moisture deficit. A measured amount of water was added to some pots twice weekly (as required by the rate of water uptake) to keep all pots at a constant weight, and then WU of each plant was recorded. For reference, samples of the soil used for filling pots, when tested on a pressure plate apparatus (Soil moisture Equipment Corp., Santa Barbara, CA) at –0.01, –0.1, and –1.5 MPa (Klute, 1986) had gravimetric soil moisture of 66, 46, and 36%, respectively. At the end of the drought period, detailed measurements of plant water relations and yield traits were carried out over a one week time window, on the stressed plants as described in Section 3.3.4 below.

3.3.4 Measurements & analyses

3.3.4.1 Plant-water relations traits

The predawn leaf water potential (LWP) of the youngest fully expanded leaf of a representative tiller of each individual PRG genotype was measured between 3 a.m. and 6 a.m. in the last week of the one-month drought period, using a Scholander's Pressure Chamber (Soil Moisture Equipment Crop., Santa Barbara. CA) (Turner, 1981). Relative water content (RWC) of stressed leaf lamina samples was calculated

using the method devised by (Barrs & Weatherly, 1962); RWC (%) = [(FW−DW)/ (TW−DW)] × 100%, where FW = leaf fresh weight, DW = dry weight of leaves after drying 80°C for 48 hours, and TW = turgid weight of leaves after soaking in water overnight in darkness at 4°C. For leaf osmotic potential (OP) measurements, fully expanded youngest leaf samples from 2 mature tillers of each PRG genotype were collected on the same morning as LWP was measured, immediately frozen in liquid nitrogen and stored at –80°C, for later measurement. Genotypes were defoliated after completion of leaf water relations measurements. The OP of frozen leaf samples was measured using a number of Wescor C-52 sample chambers (Wescor, Logan, UT, USA) by the dew-point method (Turner, 1981), in conjunction with the Wescor HR-33T microvolt meter. In order to convert the μV values to MPa, a standard curve was prepared for each chamber by measuring a series of NaCl solutions with different molarities (0.2, 0.4, 0.6, 0.8, 1.0 mol) as described in the Wescor C-52 manual (see 3A.1).

Pot WU was recorded throughout the experiment. Water-use efficiency was then calculated using pot WU and oven-dried clipped shoot dry weight (SDW) data from the end of the drought period (g WU/g SDW). At the end of the post-cutting regrowth measurement, the root system of each plant was detached (with the undisturbed soil column of each pot) from the remaining shoots and stubble and 440 genotypes were handled in four batches within a four-day time period. Roots at two different depths; (1) top root depth (RDW_T at 4–20 cm) and (2) deeper root depth (RDW_D at 20–50 cm) were then manually separated from soil using paint brushes and washed thoroughly with running water in a series of sieves (with different mesh sizes to harvest the maximum amount of cleaned roots), for root dry weight measurements. Both shoot and cleaned root samples were oven-dried separately at 80°C for 48 hours, and dry weights were taken. Leaf mass ratio (LMR; calculated as SDW/total plant DM, g/g) and root to shoot ratio (RSR; calculated as RDW/total plant DM × 100, %) were also calculated to complement SDW and RDW results (Lambers et al., 2008; Pons et al., 1998). The post-cutting regrowth or RGS (i.e. watering was completely withdrawn at the end of the drought treatment or the final defoliation) was evaluated after 7 days by using visual scores from 0 to 5, based on live tiller number as a percentage of the total tiller number (as indicated by cut tiller

bases in the stubble), as follows: 0 = 0–5%, 1 = 6–25%, 2 = 26–45%, 3 = 46–65%, 4 = 66–85%, 5 = 86–100%.

Soil moisture content (SMC) was determined by oven-drying approximately 300 g of soil collected from the 30–40 cm depth of the soil column of each pot (at the time of root harvesting) at 105°C for 48 hours. The gravimetric SMC was calculated as SMC (%, MW/MS) = (FW – DW)/DW× 100%, where FW and DW denote fresh weight and dry weight of the soil samples, respectively.

3.3.4.2 Photosynthetic gas exchange measurements

Instantaneous gas exchange variables including net photosynthetic rate (Pn), stomatal conductance (SC), leaf evapotranspiration rate (ET), leaf water vapour pressure deficit (VPD_L), relative humidity at the leaf surface (RH_L), leaf temperature (T_L), intercellular CO_2 concentration (C_i), and ratio between intercellular and atmospheric CO_2 concentration (C_i/C_a) were measured or derived from measured data under artificial, saturating photon flux density (1000 µmol/m^2/s) at an ambient CO_2 concentration of 400 ppm using a portable photosynthesis meter (LICOR 6400XT, LICOR Biosciences, Inc., Lincoln, NE, USA). Measurements were performed on two to three fully-expanded youngest leaves from representative tillers of each genotype, from 10.00–12.00 and 13.00–15.00 hours for four consecutive sunny days, Gas exchange data were recalculated according to the actual total leaf area fitted into the circular-shaped leaf chamber (diameter: 3 cm): $\sum^{n=2or\ 3}$(3 cm × (leaf width)). Because of equipment issues on one of the measurement days, gas exchange data for 38 plants could not be collected. During the period when these measurements were taken, the temperature of the leaf chamber was 26±2°C, and the relative humidity (RH) in the chamber was controlled within a range of 55–65%, similar to the RH in the glasshouse.

3.3.5 Statistical analyses

Two principal component analyses (PCA) were performed in MINITAB v.14 to identify drought-response patterns and trait associations of measured data without (PCA3.1) and with (PCA3.2) gas exchange data. Comparison of the information extracted from PCA3.1 and PCA3.2 was done by generating a correlation matrix for

the PC scores of the first six PCs of each PCA. Trait associations were also examined using Pearson's correlation analysis.

Cultivar and genotypic effects of trait means were analysed using the ANOVA model of Proc GLM in SAS v.9.4 (SAS Institute Inc., Cary, NC, USA). Comparisons of trait means among three cultivars within the population and two selected WUE groups were done using LSD mean separation test.

3.4 Results

In this chapter, the presentation of results begins with an overview of the data, setting out the mean, range and probability of cultivar and genotype differences for the measured water relations traits, gas exchange measurements, and supplementary test variables. The trait association evaluations as described in Section 3.3.1 above are then presented. Lastly, genotype groups selected for high and low WUE were compared across cultivars to understand the trait associations linked to improved drought tolerance. Some photographic comparisons of selected genotypes representing two contrasting selections are also presented for visualisation of the key results.

3.4.1 Overview of data: Within- and among-cultivar variation of the tested PRG population for measured traits

Population (collectively referring to 50, 80, and 90 genotypes (n = 220) from Nui, Samson, and Trojan cultivars, respectively) diversity was high for all measured traits and derived variables, and there were significant genotype-within-cultivar and genotype-within-population effects at $p < 0.05$ (Table 3.1 and 3.2). Moreover, significant cultivar differences were seen for most test variables, with the cultivar Trojan often separating from Samson and Nui cultivars (Table 3.1). In particular, the cultivar effect was more pronounced for SDW and WUE than for other measured traits at $p < 0.0001$ (Figure 3.2). For example, WUE of Nui, Samson, and Trojan were 419±14, 412±10, and 319.9 g WU/g DM), respectively, while values for SDW were 4.72, 4.73, and 5.75 (g/pot), respectively (Table 3.1). However, cultivar behaviour followed a different pattern for gas exchange data (i.e. Pn of Nui, Samson, and Trojan were 3.50±0.27, 4.52±0.2 and 3.62±0.19 μmol/m^2/s, respectively; Table 3.2).

Table 3.1 Cultivar and population trait means with standard error of means, and statistical probabilities of cultivar effects for Grasslands Nui (Nui), Grasslands Samson (Samson), and Trojan cultivars, and genotype effects of the tested perennial ryegrass population for morphological and water relations traits.

Trait	Nui	Samson	Trojan	p(Cv)	p(Gen)	Mean(Gen)	SEM(Gen)
SDW (g)	4.72±0.089 (1.48–6.71)	4.73±0.083 (2.51–8.67)	5.75±0.102 (2.74–10.14)	<0.0001	<0.0001	5.14	0.31
WUE (g/g)	419.14±13.41 (177.59–1046.62)	412.23±10.39 (150.29–805.50)	319.039±8.87 (70.10–983.58)	<0.0001	<0.0001	376.11	57.74
LWP (MPa)	–0.83±0.17 (–0.50 to –0.96)	–0.81±0.17 (–0.42 to –1.40)	–0.82±0.18 (–0.31 to –1.42)	<0.0001	<0.0001	–0.84	0.52
OP (MPa)	–2.10±0.052 (–1.00 to –3.69)	–2.21±0.042 (–1.30 to –4.07)	–2.49±0.052 (–1.16 to –4.52)	<0.0001	<0.0001	–2.33	0.031
RWC (%)	60.20±1.17 (30.4–88.7)	63.15±0.88 (40.3–91.8)	63.04±0.90 (36.8–89.5)	<0.0001	<0.0001	62.14	5.90
SMC_D(%)	40.04±1.45 (23.2–65.5)	40.41±0.90 (23.49–71.65)	40.92±0.83 (22.88–67.38)	<0.1000	<0.1000	40.55	13.48
RDW_T (g)	2.25±0.08 (0.11–4.31)	2.56±0.09 (0.23–8.27)	2.78±0.34 (0.34–5.81)	0.0034	<0.0001	2.58	0.82
RDW_D (g)	0.51±0.028 (0.02–1.52)	0.55±0.027 (0.00–2.33)	0.58±0.027 (0.01–1.92)	0.026	0.006	0.55	0.3
RGS	2.9±0.13 (0–5)	3.01±0.12 (0–5)	3.46±0.10 (0–5)	<0.0001	<0.0001	3.17	0.07
RDW_{Tot} (g)	2.77±0.10 (0.13–5.65)	3.11±0.11 (0.24–9.10)	3.37±0.10 (0.39–7.64)	0.0065	<0.0001	3.13	1.03
PDW (g)	7.49±0.15 (1.16–10.11)	7.83±0.16 (3.04–15.94)	9.12±0.16 (3.53–17.78)	<0.0001	<0.0001	8.28	1.09
LMR (%)	61.68±0.91 (43.1–91.92)	61.62±0.78 (38.24–92.92)	63.85±0.75 (30.73–91.42)	0.0211	0.0112	63.13	8.28
RSR (%)	58.88±2.22 (8.78–132.01)	64.58±2.19 (7.62–161.52)	66.72±2.42 (9.38–225.41)	0.0196	<0.1000	62.39	23.34

*SDW, Shoot dry weight; WUE, Water-use efficiency (g WU/g DM); LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC_D, gravimetric soil moisture content at 30–40 cm depth (w/w); RDW_T, Root dry weight at 4–20 cm depth; RDW_D; RGS, Post-cutting regrowth score; Root dry weight at 20–50 cm depth; RDW_{Tot}, Total RDW; PDW, Total plant dry weight; LMR, Leaf mass ratio; RSR, Root to shoot ratio; p(Cv), Statistical probability of cultivar effect; p(Gen), Statistical probability of genotype-within-population effect; SEM, Standard error of mean (Note: The range among genotypes-within-cultivars appear in parentheses).

Table 3.2 Cultivar and population trait means, standard error of means, and statistical probabilities of cultivar effects for Grasslands Nui (Nui), Grasslands Samson (Samson), and Trojan cultivars, and genotype effects of the tested perennial ryegrass population for gas exchange measurements. The range among genotypes-within-cultivars appears in parentheses.

Trait	Nui	Samson	Trojan	p(Cv)	p(Gen)	Mean(Gen)	SEM(Gen)
Pn (μmol/m^2/s)	3.50±0.27 (0.33–9.81)	4.52±0.20 (0.44–11.16)	3.62±0.19 (0.18–10.43)	<0.0001	<0.0001	3.92	0.13
Ci (μmol/m^2/s)	263.34±1.88 (220.31–299.90)	262.30±1.61 (222.52–307.14)	270.27±1.68 (226.37–367.75)	<0.0001	<0.0001	265.83	1.06
Ci/Ca	0.79±0.005 (0.66–0.89)	0.79±0.004 (0.67–0.91)	0.80±0.0006 (0.68–0.92)	<0.0001	<0.0001	0.79	0.003
SC (mmol/m^2/s)	0.051±0.002 (0.007–0.100)	0.054±0.0003 (0.013–0.097)	0.052±0.001 (0.009–0.095)	<0.0001	<0.0001	0.048	0.012
ET (mmol/m^2/s)	2.40±0.07 (1.21–4.49)	2.77±0.08 (1.11–4.87)	2.47±0.06 (1.11–4.72)	<0.0001	<0.0001	2.57	0.043
T_L (ºC)	26.16±0.13 (24.02–29.26)	25.78±0.09 (23.17–27.98)	25.99±0.086 (22.84–29.07)	0.0004	<0.0001	26.01	0.058
VPD_L (kPa)	1.13±0.03 (0.79–2.84)	1.17±0.02 (0.81–1.50)	1.14±0.014 (0.81–1.50)	<0.0001	<0.0001	1.17	0.11
RH_L (%)	50.43±0.20 (46.53–54.9)	51.03±0.17 (47.17–56.03)	51.09±0.17 (47.48–57.10)	<0.0001	<0.0001	50.91	0.13

Trait abbreviations: Pn, Photosynthesis; Ci, Intercellular CO_2 concentration; Ci/ Ca, The ratio between Ci and atmospheric CO_2; SC, Stomatal conductance; ET, Evapotranspiration; T_L, Leaf temperature; VPD_L; Leaf vapor pressure deficit; RH_L, Leaf relative humidity; P(Cv), Statistical probability of cultivar effect; P(Gen), Statistical probability of genotype-within-population effect; SEM, Standard error of mean.

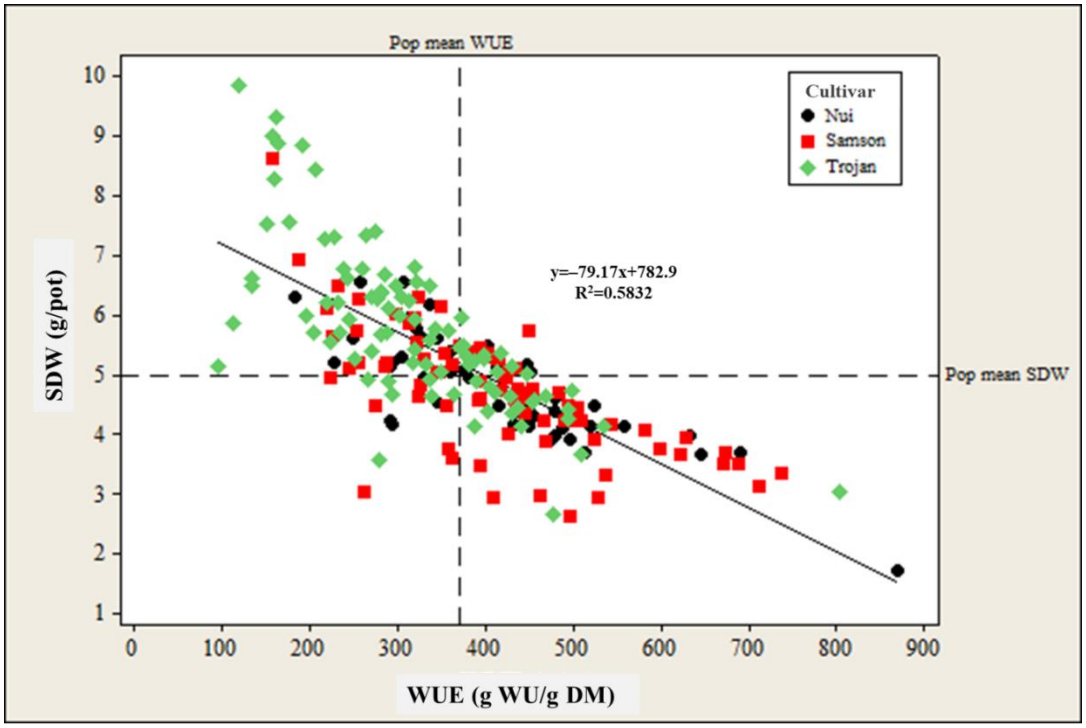

Figure 3.2 Graphical representation of the distribution of shoot dry matter production (SDW; g/pot) against the amount of water consumed per unit of dry matter production (WUE; g WU/g DM) of three perennial ryegrass cultivars; Nui, Samson and, Trojan within the tested population.

3.4.2 Key drought-response patterns and beneficial traits associations

The first PCA (PCA3.1) was compiled for data of nine water relations and morphology trait measurements and the first four PCs explaining 76% of total variation (eigenvalue > 0.9 (Jolliffe, 2003)) were selected for interpretation. PC1 explained 32.7% of the total variance, while PCs 2, 3, and 4 explained 19.5%, 13.4% and 10.3%, respectively (Table 3.3). PC1 was represented by a plant type with faster growth rate associated with greater extraction of soil moisture due to the presence of a deep and dense root system (i.e. positive coefficients for both RDW_T and RDW_D). PC2 identified a plant type that maintained high SDW through increased WU (but lower RDW_D) and lower leaf hydration indicative of high evapotranspiration. PC3 elucidated a plant type that maintained improved leaf hydration and high SDW high RDW_D with low WU and soil moisture conservation (positive coefficient for SMC). PC4 indicated that RGS (coefficient –0.858) was independent of the other measured traits to a large extent. Amongst all four PCs, the plant type identified by PC3 was notable for having a higher RGS on re-watering

after drought than plants with high SDW that were identified by PC1 or PC2, with an indication of OA (i.e. high RWC at more negative OP and LWP).

Table 3.3 Principal component (PC) coefficients for the first four PCs generated by PCA3.1 of nine water relations traits of 220 means of 440 perennial ryegrass genotypes comprised three cultivars; Nui, Samson and, Trojan.

	PC 1	**PC 2**	**PC 3**	**PC 4**
Eigenvalue	2.94	1.76	1.21	0.93
% variation explained	32.7	19.5	13.4	10.3
Cumulative % variance	32.7	52.2	65.6	75.9
SDW	0.380	0.299	0.476	0.139
WUE	–0.362	0.380	–0.424	–0.186
LWP	–0.212	0.273	–	0.162
OP	–0.314	0.513	–0.130	–0.300
RWC	–	–0.598	0.246	0.324
SMC_D	–0.395	–0.128	0.383	0.184
RDW_D	0.423	–0.115	0.430	–
RDW_T	0.484	–	–0.195	–
RGS	–	–0.195	0.382	–0.858

Trait abbreviations: SDW, Shoot dry weight; WUE, Water-use efficiency (g WU/g DM); LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC, gravimetric soil moisture content at 30–40 cm depth; RDW_T, Root dry weight at 4–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth; RGS, Post-cutting regrowth score. (Note: Negative coefficients for WUE indicate less WU/g DM).

To further explore the properties of the current data set when analysed by PCA, instantaneous gas exchange and related data captured by the LiCor portable

photosynthesis meter (LiCor 6400XT, LiCor Inc., USA) were added to the data matrix submitted to PCA3.1 to form PCA3.2 based on 16 plant morphology, water relations, and gas exchange traits as indicated in Section 3.3.5 above.

In PCA3.2, 72.5% of the total data variation was explained by the first six PCs of the total of 16 PCs, with eigenvalue > 0.9 (Jolliffe, 2003) (Table 3.A1). In particular, it was found that the introduction of gas exchange data in PCA3.2 caused a 'reorganisation' of the information extracted by PCA3.1 from the measured data above. Therefore, to visualise how information in the data of PCA3.1 was statistically reorganised by the inclusion of gas exchange data, a correlation analysis was performed for the PC scores of first four PCs of PCA3.2 against those of PCA3.1 (Table 3.A2). It was seen that PC1 explaining 32.7% of the total data variation in PCA3.1 was split into two PCs in PCA3.2 with the introduction of additional trait data (i.e. PC1 and PC2 together explained 39.7% of the total variance in PCA3.2), but with a similar alignment of both PCs with PC1 of PCA3.1 ($r_{PC1\text{-}PC1}$ = 0.985 and $r_{PC2\text{-}PC1}$ = 0.976, respectively). By definition, PC1 and PC2 were uncorrelated with each other, particularly with contrasting LWP and gas exchange properties including SC in PCA3.2. PC2 of PCA3.1 was clearly aligned with PC3 of PCA3.2 (r=0.994) while PCs 2 and 3 of PCA3.2 identified less-stressed genotypes (i.e. positive coefficients for LWP, Pn, and SC). There was a lesser alignment of PCs 3 and 4 of PCA3.1 with those of PCA3.2. However, PC scores of PC4 of PCA3.2 showed the closest alignment with those of PC3 in PCA3.1(r=0.650) (Table 3.A2). However, both cultivar-wise and population-wise simple correlations between the key traits were less-informative (data are not presented).

3.4.3 Divergent selection

The trait association of the plant type that was represented by PC3 of PCA3.1 (Table 3.3) was identified as being of interest for further study. Hence, a divergent selection was made, based on PC3 scores. Accordingly, two divergent groups; high g WU/g DM (i.e. 15 LWUE genotypes: 7, 5, and 3 genotypes from Nui, Samson, and Trojan cultivars, respectively) and low g WU/g DM (i.e. 20 HWUE genotypes: 5, 7, and 8 genotypes from Nui, Samson, and Trojan cultivars, respectively) were selected from the 220 PRG genotypes. It was salient to note that HWUE genotypes exhibited higher SDW, RDW_D, and high SMC at 30–40 cm soil depth together with more

negative OP compared to those of LWUE genotypes, as expected. For example, HWUE genotypes of Nui, Samson, and Trojan cultivars showed increments of 46%, 48%, and 60%, respectively, in SDW compared to the average SDW of LWUE genotypes of the three cultivars (Figure 3.3). Water-use efficiencies averaged for the HWUE and LWUE groups were 301.05±36 g WU/g SDW and 550.90±36 g WU/g SDW, respectively, displaying a 1.8-fold difference between the two groups for WUE (Figure 3.3).

The cultivar Trojan often separated from Samson and Nui cultivars for most traits measured under imposed drought (i.e. values of OP averaged for HWUE genotypes of Nui, Samson, and Trojan cultivars were –2.7, –2.8, and –3.2 MPa, respectively). Also, for RWC, RDW_D, and SMC traits, HWUE genotypes of Trojan cultivar exhibited higher RDW_D, RWC, and SMC at 30–40 cm soil depth, compared to HWUE genotypes of Nui and Samson cultivars (Figure 3.4). However, cultivar effects were less significant for traits related to morphological plasticity (i.e. LMR and RSR) in both WUE groups even though the differences in average LMR and RSR between HWUE and LWUE groups were significant at $p<0.05$ (Figure 3.5).

Both cultivar and WUE group effects for gas exchange data were less significant at $p>0.05$ (Figure 3.6). However, it was important to note that HWUE genotypes of the cultivar Trojan showed the lowest average SC and ET and the highest average Ci among the three cultivars with an average performance of Pn under imposed drought (Figure 3.6).

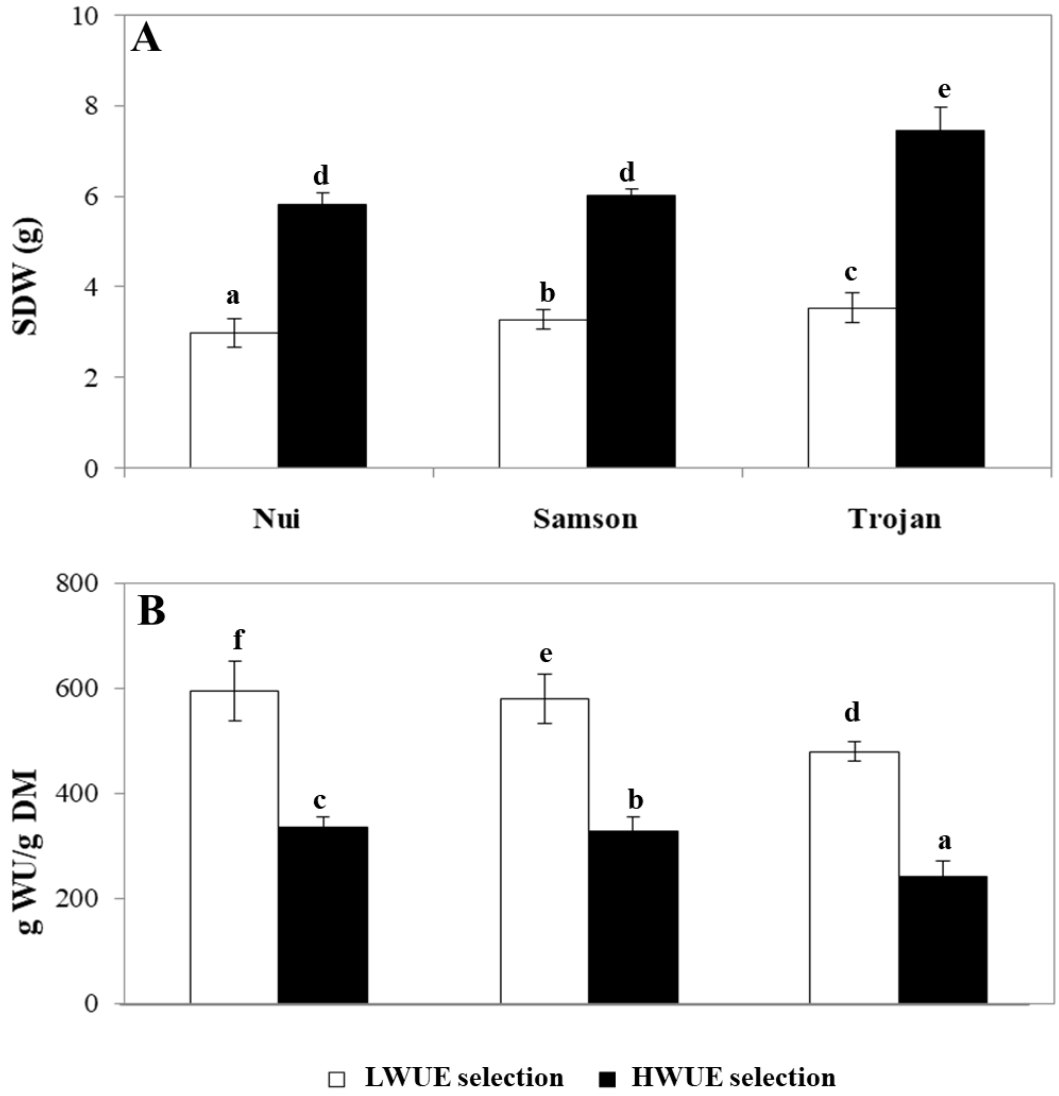

Figure 3.3 Means for (A) shoot dry matter (SDW) and (B) water use per gram of shoot dry matter (g WU/g DM) for divergently selected groups of genotypes within the cultivars. Error bars are LSDs for cultivar trait means of HWUE and LWUE selections; Bars with letters in common do not differ statistically (p = 0.05) and vice versa in the ANOVA. (Abbreviations: LWUE, Low water-use efficiency; HWUE, High water-use efficiency).

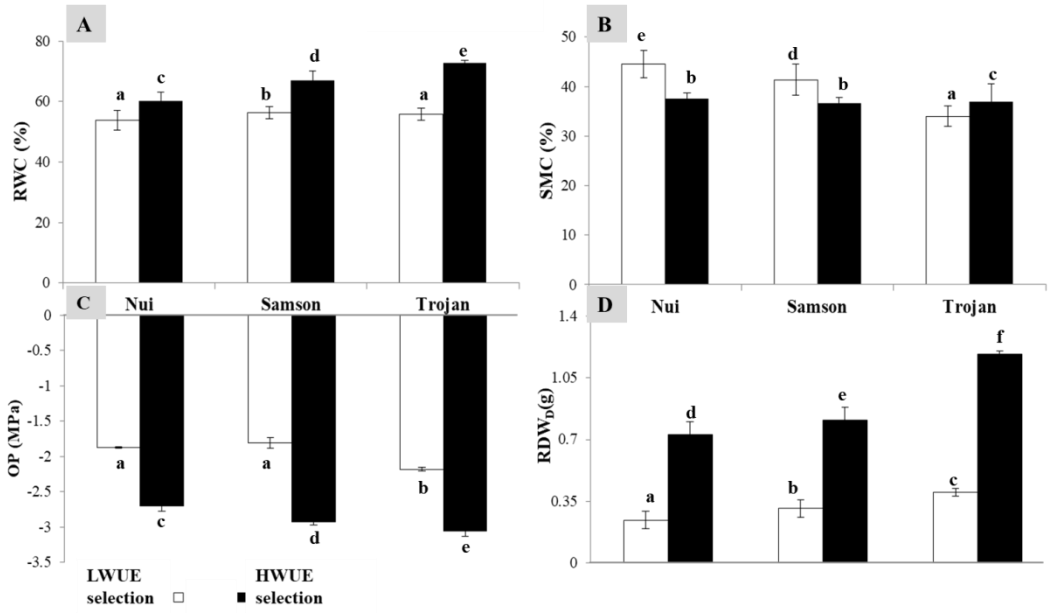

Figure 3.4 Average (A) leaf relative water content (RWC), (B) gravimetric soil moisture content at 30–40 cm soil depth (SMC), (C) leaf osmotic potential (OP), and (D) root dry weight at 20–50 cm depth (RDW_D) within cultivars of divergently selected groups (i.e. HWUE and LWUE). Error bars are LSDs for cultivar trait means of HWUE and LWUE selections; Bars with letters in common in each graph do not differ statistically (p = 0.05) and vice versa in the ANOVA.

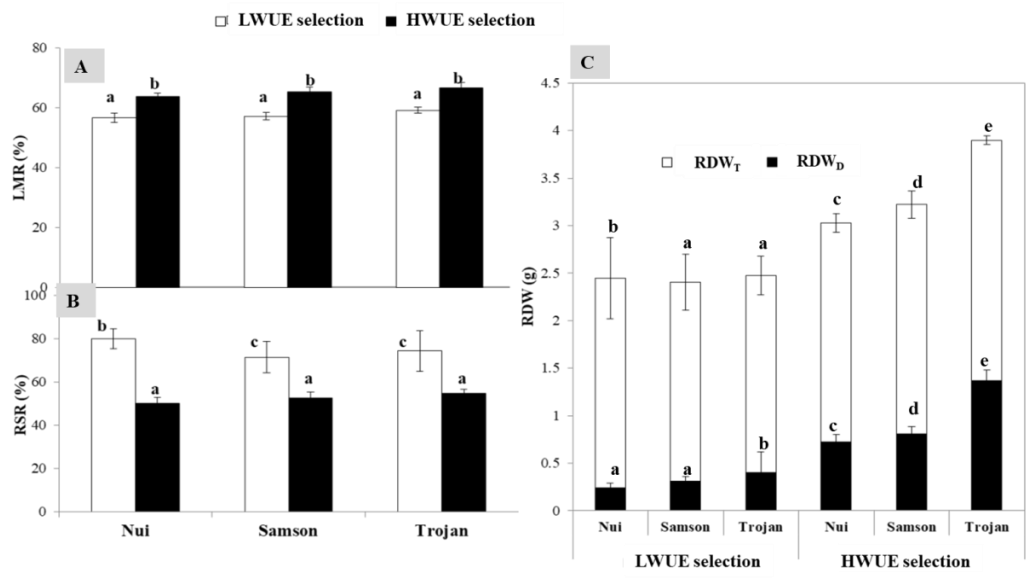

Figure 3.5 Average (A) leaf mass ratio (LMR), and (B) total root: shoot ratio (RSR), and (C) proportions of root biomass at two root depths (i.e. RDW_T, Root dry weight at 4–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth) within cultivars of divergently selected groups (i.e. HWUE and LWUE). Error bars are LSDs for cultivar trait means of HWUE and LWUE selections; Bars with letters in common in each graph do not differ statistically (p = 0.05) and vice versa in the ANOVA.

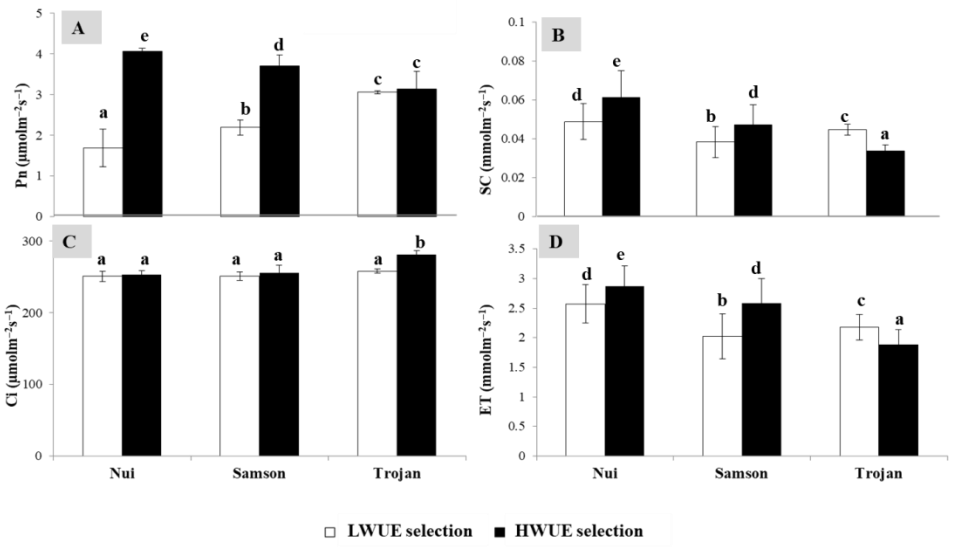

Figure 3.6 Average (A) net photosynthesis (Pn), (B) stomatal conductance (SC), (C) intercellular CO_2 concentration (Ci), and (D) leaf evapotranspiration (ET) within cultivars of divergently selected groups (i.e. poor and good performers) under imposed drought. Error bars are LSDs for cultivar trait means of HWUE and LWUE selections; Bars with letters in common in each graph do not differ statistically (p = 0.05) and vice versa in the ANOVA.

3.4.4 Photographic comparisons of divergently selected genotypes within cultivars Nui, Samson, and Trojan

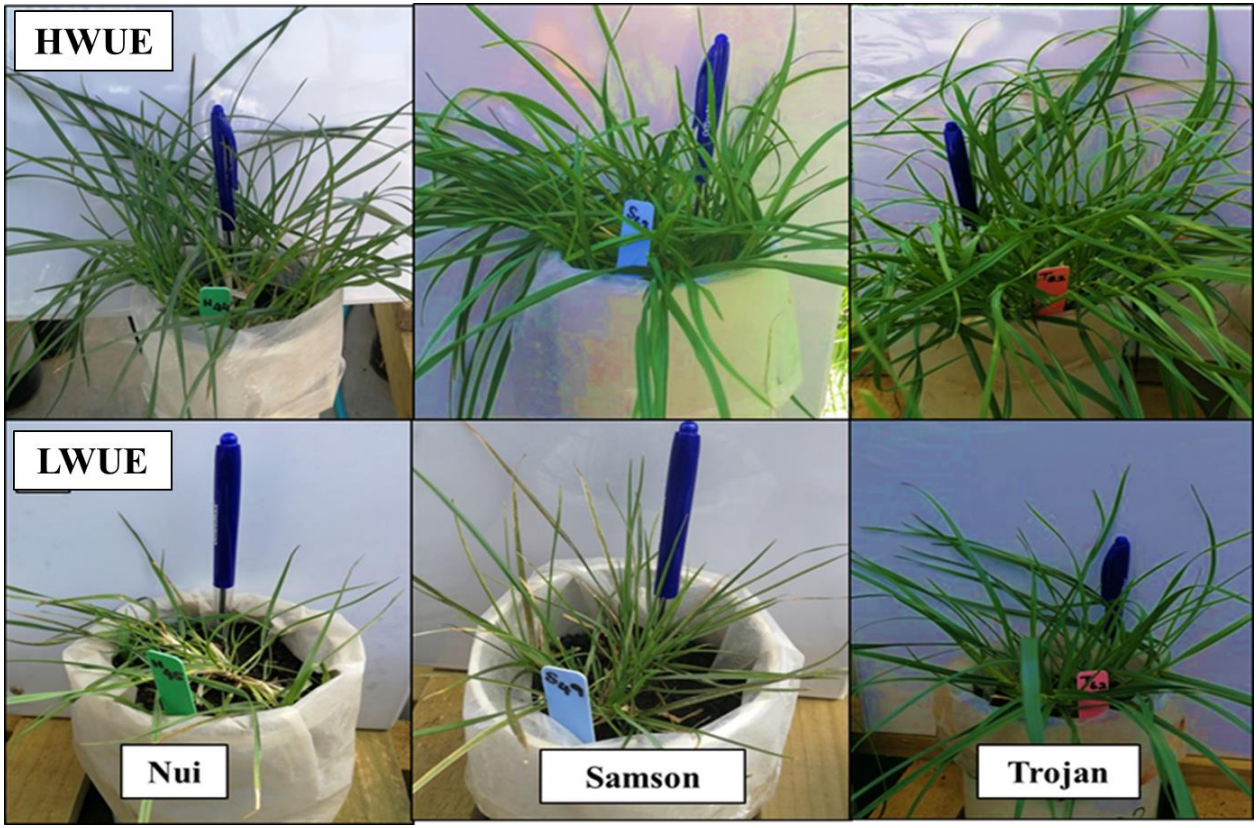

Figure 3.7 Visual representation of HWUE and LWUE canopy morphology in Experiment 1 (Note: 'HWUE' and 'LWUE' denote low and high water use perennial ryegrass genotypes, respectively, and photographs were taken before imposing drought).

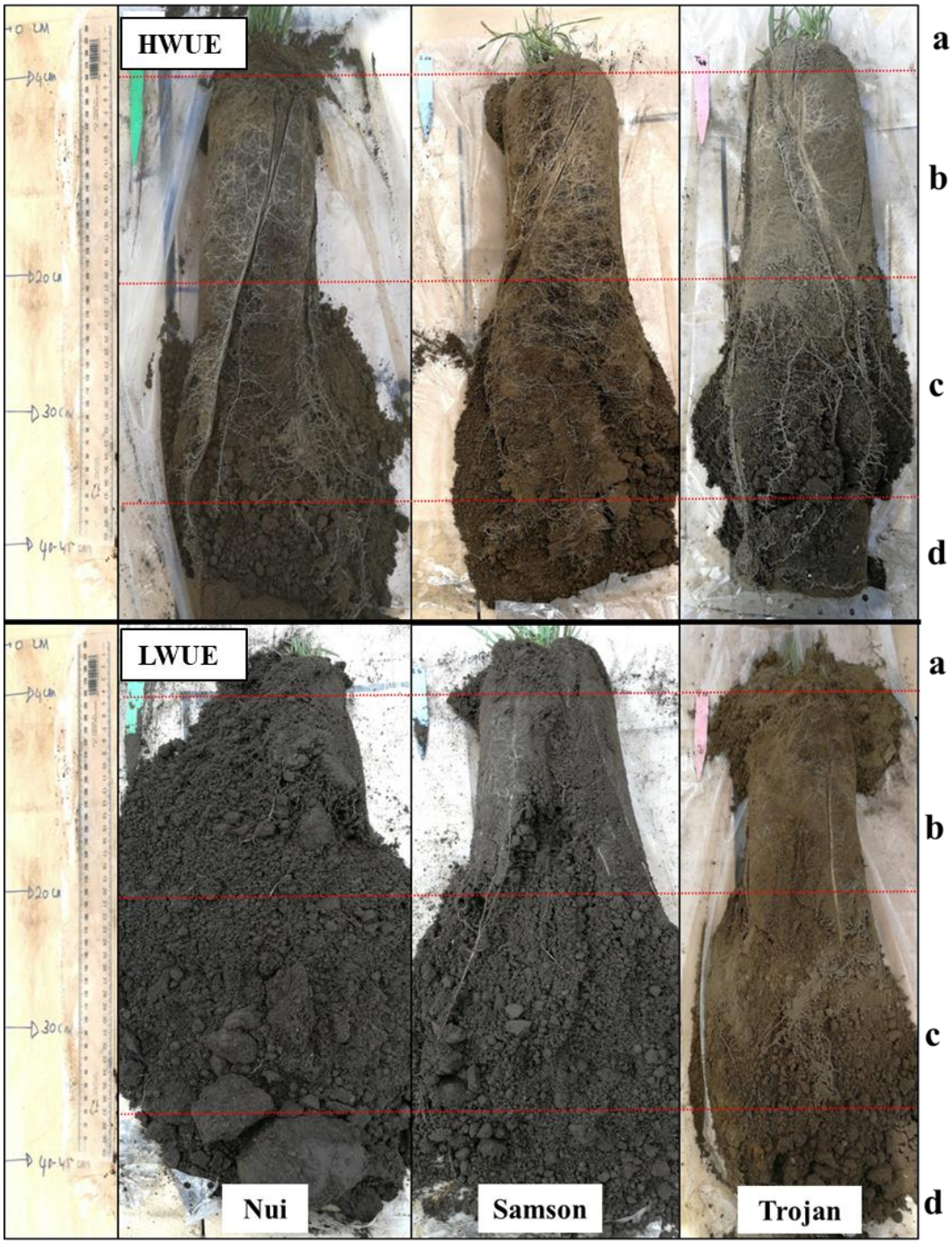

Figure 3.8 Contrasting genotypes (HWUE and LWUE, low and high water use genotypes, respectively) of three perennial ryegrass cultivars (Nui, Samson, and Trojan) demonstrating the difference in rooting behaviour and consequent soil moisture retention at the end of the imposed drought challenge. (Note: a = removed crown part, b = top roots at 0–20 cm, c = deep roots at 20–50 cm and, d=sampling depth for post-drought soil moisture measurements: 30–40 cm) (see Table 3.4 for reference data below).

Table 3.4 Data on RDW_T, RDW_D, RSR, and SMC traits for the photographed plants in Figure 3.8.

	Nui		Samson		Trojan	
	HWUE	LWUE	HWUE	LWUE	HWUE	LWUE
RDW_T (g)	2.15	2.80	2.45	2.40	2.73	2.07
RDW_D (g)	0.78	0.25	0.60	0.32	1.04	0.22
RSR (%)	51	77	57	77	51	67
SMC (%)	38	45	36	39	36	35

Trait abbreviations: RDW_T, Root dry weight at 0–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth; RSR, Total root to shoot ratio; SMC, gravimetric soil moisture content at 30–40 cm depth (w/w); HWUE and LWUE, low and high water use genotypes, respectively.

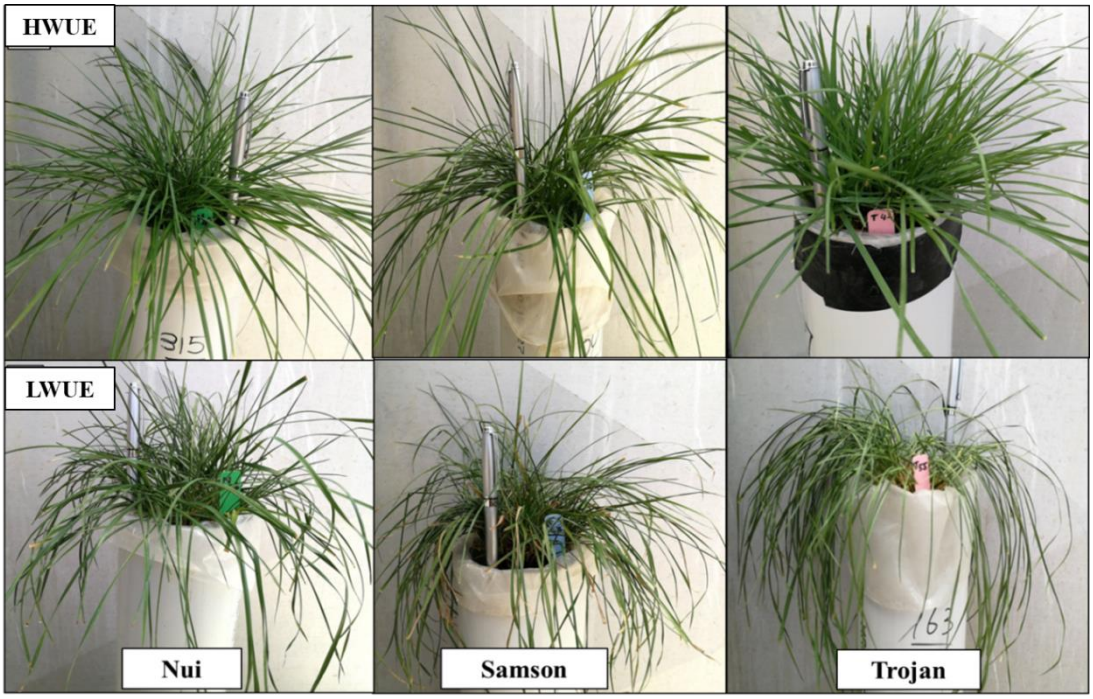

Figure 3.9 Visual differences between high and low water use genotypes (i.e. LWUE and HWUE genotypes, respectively) within populations of Nui, Samson and, Trojan cultivars under imposed drought (Note: Genotypes with high water use (i.e. LWUE) needed more water for the same DM yield with less degree of wilting and leaf senescence, had reduced water-use efficiency and vice versa).

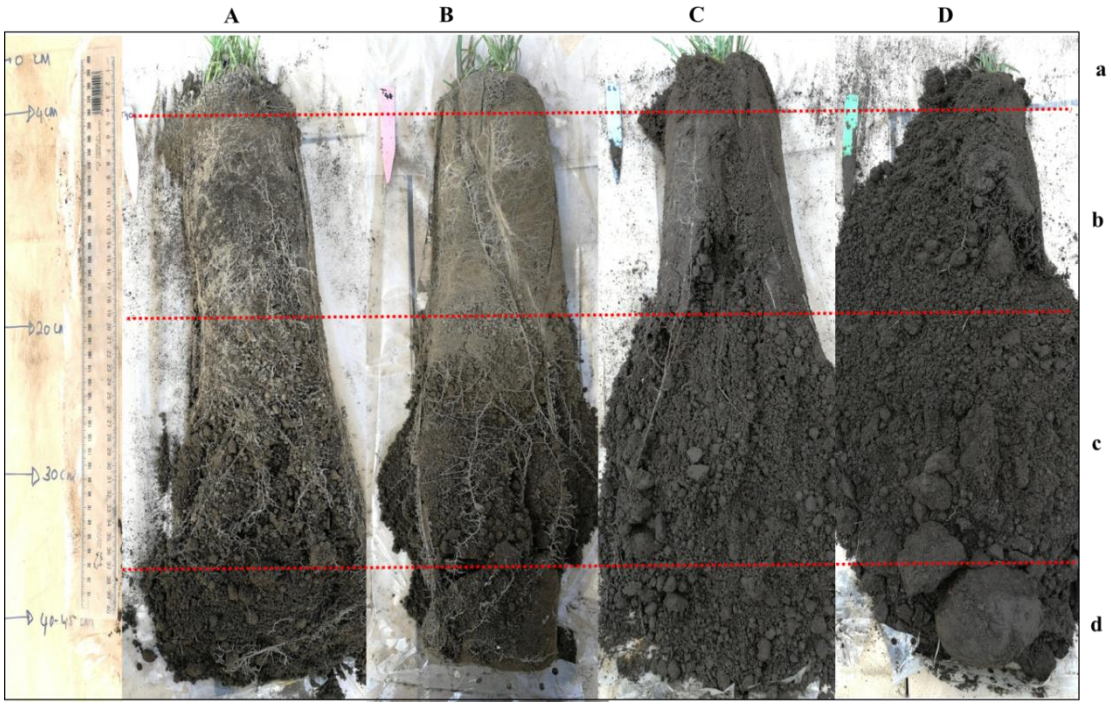

Figure 3.10 Four divergently selected genotypes (A and B, low water use genotypes; C and D, high water use genotypes) representing differential deep rooting behaviour that contributed to the trait associations identified in PCA (see Table 3.5 below for reference data from the photographed plants).

Table 3.5 Data on RDW_D, OP, RWC, SDW, SMC, and RGS for the photographed plants in Figure 3.10.

	A	**B**	**C**	**D**
RDW_D (g)	1.88	1.04	0.32	0.34
OP (MPa)	–3.12	–3.07	–1.84	–1.77
RWC (%)	64	63	59	51
WUE (g/g)	119.50	273.30	610.83	697.26
SDW (g)	9.85	7.40	3.10	2.70
SMC (%)	30	33	40	43
RGS (1–5) scale	4	4	3	2

Trait abbreviations: RDW_D, Root dry weight at 20–50 cm depth; OP, Leaf osmotic potential; RWC, Leaf relative water content; WUE, Water-use per gram of DM production; SDW, Shoot dry weight; SMC, gravimetric soil moisture content at 30–40 cm depth (w/w); RGS, Post-cutting regrowth score (Note: a = removed crown part to maintain planting materials for the next experiment above 4 cm depth, b = top roots at 4–20 cm, c = bottom roots at 20–50 cm and, d = sampling depth for post-drought soil moisture measurements: 30–40 cm).

Figure 3.11 Water deficit response of four reference plants: (a) A perennial ryegrass genotype grown in soil at near field capacity: LWP and OP, –0.3 MPa and –0.8 MPa, respectively; (b) A high water use genotype exposed to the severe drought challenge: LWP and OP, –1.0 MPa and –1.8 MPa, respectively; (c) A low water use genotype exposed to the severe drought challenge: LWP and OP, –1.0 MPa and –2.8 MPa, respectively; (d) A perennial ryegrass genotype kept at the permanent wilting point: LWP and OP, –3.6 MPa and –3.4 MPa, respectively.

3.5 Discussion

The intention was for genotypes of Nui, Samson, and Trojan cultivars to be exposed to a moisture deficit challenge of sufficient duration and intensity to display their drought tolerance attributes. Previous studies (He, 2016; Hussain, 2013) and a pilot study (see Appendix 1A.1) showed that a drying down period of 3–4 weeks to reach a LWP of approximately –0.9 MPa meets these criteria. Clipped PRG genotypes at the start of dry-down were estimated to have had about 20–25 days' supply (1808 g SMC/ 73 g/day ET = 25 days; this may differ according to the growth rate of each genotype) of soil-held, plant-available water in pots. To obtain this piece of information in potted PRG genotypes, basic estimates including soil available moisture (including pot weight), plant evapotranspiration, and WHC of the test soil were recorded at the beginning of the experiment. Plant ET recorded for a single clipped PRG genotype was 68–78 g/day (73 g/day on average), with 1300 g of soil moisture available per pot (average weight of an empty pot was 350 g) at FC that was held by 3800 g of dry soil (i.e. total pot weight at FC = 1300 + 3800 + 350 g = 5450 g). Soil moisture available between FC and 50% PWP until the next irrigation was calculated as; SMC = 1300 + (1300 g/46%) ×36%×0.5 =1808 g, where gravimetric soil water available at FC and PWP were 46% and 36% MW/MS (Section 3.3). Soil water storage was boosted by choice of 'A' horizon of an 'Egmont Black Loam' (a Typic Distrandept under USDA nomenclature, (Perrott & Sarathchandra, 1987)) with high WHC (46% MW/MS of soil water available at FC) as a soil medium for filling pots.

3.5.1 Water relations traits including WUE as selection criteria for PRG drought tolerance

There was a high heterogeneity within- and among-cultivars in the source population (Table 3.2 and 3.3), and simple correlation analysis was unable to identify different sets of PRG genotypes with different trait associations within the source population in the way PCA could. Therefore, in the current experiment, PCA was used to explore the data for different drought response patterns of measured traits in different subsets of the 220 PRG genotypes evaluated under imposed drought.

3.5.1.1 Population variation for WUE

In order to design an effective plant breeding program, it is important to gain knowledge about population mean and genotypic diversity of traits of interest in the source population that is to be used as the base material for the development of a new cultivar through recurrent selection (Section 2.6.2; (Acquaah, 2012)). Results of Experiment 1 indicated a suitably high population mean including high cultivar means for SDW and WUE under imposed drought (Table 3.2). Moreover, the large within-population and within-cultivar variation observed for measured traits in the current experiment demonstrated the suitability of the population to undergo selection for traits of interest (Tables 3.2 and 3.3). Visual comparisons of representative plants observed for the variations in shoot morphology (Figure 3.7), root morphology (Figure 3.8), and pot WU (Figure 3.9) further illustrate the existing genotypic variation for the key traits. One explanation for the evident drought tolerance of the tested material may be the history and breeding background or germplasm origins of the three cultivars tested. The cultivar Nui was derived from an old pasture 'Mangere ecotype', which is known to be better adapted to periodic summer moisture deficit conditions and high summer temperatures (Corkill et al., 1981). The cultivar Samson was bred for high summer productivity using Nui as one of the parents (Stewart, 2006). The cultivar Trojan was bred with the introgression of Spanish PRG germplasm to the New Zealand germplasm targeting improved summer yield and persistence (Easton et al., 2011). The high within-cultivar variability observed for measured traits can be attributed to the fact that PRG is a cross-pollinated species that normally possesses a high genetic variability for morpho-physiological traits within a given population (Cui et al., 2015; Thorogood, 2003) and lack of previous selection for the drought tolerance traits within these populations.

3.5.1.2 Trait response patterns of WUE

Previous opinion has held that selection approaches aimed at improved performance of plant physiological traits that contribute to both survival and production under water-limited environments are most likely to contribute to yield improvements under drought (Blum, 2005; Richards, 1982, 1996). Also, WUE, as a productivity trait, may assist the breeder to select elite plants with high yields under drought (de

Almeida Silva et al., 2012; Feldman et al., 2018; Ferguson et al., 2018). However, WUE has been neglected in crop breeding programs and this trait has seldom been discussed in pasture breeding programs.

It has previously been found that high WUE is primarily equivalent to reduced WU that is also a trait linked to yield reduction in a range of drought-response patterns triggered under drought stress (Blum, 2005; Blum, 2009). For that reason, the author's approach was to focus on an ideal combination of drought tolerance traits rather than focusing on WUE as a single trait in a selection program. There was a high heterogeneity within- and among-cultivars in the source populations (Tables 3.1 and 3.2 and Figures 3.7–3.10) that contributed to grouping of plants by PCA. Thus, results of both PCAs 3.1 and 3.2 identified different patterns of trait associations related to WUE. For example, it was clear that the large majority of genotypes tested exhibited a trait association consistent with the WUE approach advocated by Blum (2009) and characterised by Blum as 'effective use of water' or 'EUW' (Section 2.2.4.1). Such a plant type may use more negative leaf OP as a signal to extend roots to search for water from drying soils causing greater extraction of soil moisture (negative coefficient for SMC) and in that way maintain net assimilation and growth, which may well have contributed to high WUE of that plant type in the current experiment (Table 3.3 and 3.A1). It was important to note that a minority of genotypes (PC3 of PCA3.1; Table 3.3) exhibited strongly negative OP, leaf hydration, and higher regrowth rate (indicative of OA) but average deep rootedness and slower soil moisture depletion that led to improved WUE. The latter drought response pattern had a conceptually ideal trait association for PRG drought tolerance and visual inspection of genotypes identified by scores of PC1 of PCA3.1 confirmed the fact that this specific trait response related to 'true WUE' is easily recognisable. Therefore, the grouping generated by PC1 of PCA3.1 appears to provide a useful discrimination between plant types for breeding purposes.

The constructive relationship between OA (derived from OP and RWC data) (Cyriac et al., 2018) or deep rootedness (Johnson & Asay, 1993) and regrowth has found in in high-yielding PRG accessions (Bothe et al., 2018; Cyriac et al., 2018) and also in drought tolerance cultivars of major field crops (Abid et al., 2018; Arab et al., 2019; Blum, 1989; Blum, 2017; Manavalan et al., 2009) in previous studies and under imposed drought. For example, Blum (2005) has listed 22 studies that evaluated the

relationship between drought resistance, OA, and yield in ten crops, that discussed the role of OA in both plant survival and yield maintenance under water deficit. The same review also noted evidence of the contribution of OA to moisture extraction from deeper soil layers, indicating efficient water uptake in sorghum. Moreover, a recent review, that examined 24 studies to compare OA with yield gain in variable genotypes in 12 field crops under imposed drought, found a positive and significant association between OA, deep rootedness, regrowth, and yield (Blum, 2017). However, comparable information in a few other reports was equivocal (Blum, 2017) and such studies suggested that OA is merely advantageous for the post-drought plant recovery and plant survival under intense drought, which conflicts with high yield gain of a given plant (Blum & Tuberosa, 2018; Thomas & Evans, 1989). Perennial ryegrass experiments where OP provided dubious benefits have been carried out in sand-based media with low WHC or under mild water deficit (LWP: –0.7 to –0.8 MPa, OP: –1.7 to –1.9 MPa) (Thomas & Evans, 1989). It can be assumed that rapid dry-down of sandy potting media might not have allowed time for intracellular physiological adjustment followed by osmoregulation, or less severe moisture deficit might not have fully stimulated it. The current study involved a moderate to severe dehydration cycle (LWP ranged from –0.9 to –1.3 MPa and OP fluctuated within a range from –2.0 to –4.5 MPa), with a slow dry-down occurring over four weeks as a result of the high WHC of the Egmont silt loam soil used for the potting medium. Therefore, the possibility of inducing genotypes to express any genetic potential for OA may well have enhanced in the current experiment (Figure 3.10). As a result, a subset of PRG genotypes (PC3 of PCA3.1) with high SDW and WUE exhibited more negative OP and higher plant hydration at low LWP, which is taken here to be indicative of OA.

Despite the research attention given to the trait associations between OP or OA, deep rootedness, and SDW of PRG, the role of those trait associations in keeping high WUE of drought-tolerant grass species has seldom been documented, although some studies have explored the relationship between OA and intrinsic WUE (in terms of instantaneous WUE or carbon and oxygen isotope discrimination (Ebdon & Kopp, 2004; Ghannoum et al., 2002) and findings were inconclusive. For example, Hessini et al. (2009) asserted that OA and instantaneous WUE were differently associated in moderate (50% FC) and severe (25% FC) drought conditions, which

led to only a partial contribution of OA to WUE or moisture deficit tolerance in smooth cordgrass genotypes. One study found that genotypic differences exhibited for intrinsic WUE in alfalfa at 50% FC was closely associated with those observed for antioxidant properties of OA under imposed drought (He et al., 2012). However, it is possible that the instantaneous proxy measures of WUE would not fully reflect actual WUE integrated over a period of time. Even so, a comparison between intrinsic or instantaneous WUE and actual WUE was not made in the current experiment. Nevertheless, soil water balance and biophysical models recommended for estimating pasture irrigation budgets are based on the actual crop water usage and they also focus on the associated properties such as shoot regrowth, plant water status, root depth and distribution, and soil moisture characteristics (Johnson et al., 2008; Truter et al., 2016). Therefore, the trait behaviour of the potential subset of genotypes (as represented by PC1 of PCA3.1; Table 3.3) in this experiment merits further study in order to establish the consistency of the response in different moisture deficit challenge events at different times.

Another reassuring feature of the results of the current experiment was that data checks of the key traits indicated a good agreement between the two replicates of each genotype. For example, the correlation estimates between replicates for SDW, WUE, and OP traits among the 220 genotypes tested were, respectively, 0.943, 0.861, and 0.805 ($p < 0.001$). This engenders confidence that the test methodology can provide results that are sufficiently consistent for plant breeding purposes, if WUE and the key traits measured are heritable, and if the moisture deficit challenge is sufficiently representative of field conditions faced by plants in summer.

3.5.2 The cultivar Trojan had the strongest WUE-OP trait association of the tested PRG cultivars

3.5.2.1 Cultivar ranking for WUE

The three PRG cultivars representing a time series of release dates; Nui = 1970s, Samson = 1990s and, Trojan = 2000s showed a significant cultivar-wise difference for most traits, with the cultivar Trojan separating from the other two cultivars (Table 3.1 and 3.2). In particular, for divergently selected groups, the cultivar ranking Trojan > Samson > Nui was more pronounced for SDW and WUE in the HWUE group and with parallel movement in associated traits including OP and

RDW_D (Figure 3.3–3.5). Thus, the yield increase in HWUE genotypes of Trojan compared to that of Nui was closely associated with increased WUE and OP, assuming plants dried the soil down to a similar soil WP across the test genotypes within the population. A similar result was also seen in the pilot study conducted in summer of 2014/2015 (see 1A.1). Hence, the cultivar ranking Trojan> Samson> Nui for WUE appears to indicate that four decades of plant breeder selection for yield has resulted in concurrent improvement in WUE, and that the intended improvement in tolerance of summer temperatures and moisture deficit through incorporating Spanish germplasm (i.e. drought-avoidant characteristics) into modern PRG cultivars (Easton et al., 2011; Stewart, 2006) has been achieved.

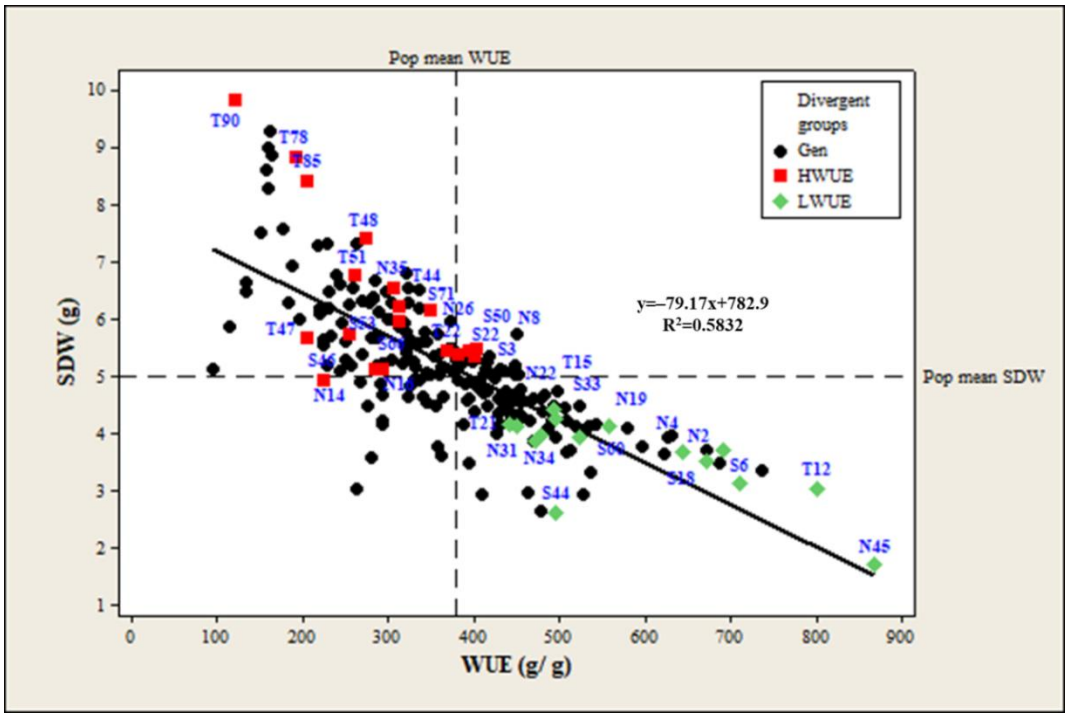

Figure 3.12 Graphical representation of the distribution of shoot dry matter production (SDW; g/pot) against the amount of water consumed per unit of dry matter production (WUE; g WU/g DM) of two divergent groups; low water use (HWUE) and high water use (LWUE) within the tested perennial ryegrass population (Note: N, S, and T letters represent selected genotypes (Gen) of Nui, Samson, and Trojan cultivars, respectively, within HWUE and LWUE groups and they are genotype identifiers together with numbers in blue colour).

3.5.2.2 Cultivar effects of the 'SMC-conserving' WUE-OP-RDW_D trait association of elite PRG genotypes

Cultivar effects of measured traits (i.e. within population and HWUE and LWUE groups) can be seen by the comparative inspection of results in Table 3.1 (i.e. morphological and water relations traits) and Table 3.2 (i.e. gas exchange measurements) together with Figure 3.3 (i.e. (A) SDW and (B) WUE), Figure 3.4 (i.e. (A) RWC, (B) SMC, (C) OP, and (D) RDW_D), and Figure 3.6 (i.e. (A) Pn, (B) SC, (C) ET, and (D) Ci). Accordingly, OP became more negative across the cultivar time series of 'Nui'-'Samson'-Trojan and simultaneously, RWC, WUE, SDW, and RDW or RSR (including both RDW_D and RDW_T) showed an increase at a stable LWP (i.e. –0.9 MPa). In the HWUE group, only Trojan had elite genotypes that displayed high RWC (i.e. more than 60%) and strongly negative OP (i.e. less than –3.5 MPa) together with the lowest LWP values (i.e. less than –1.3 MPa) under imposed drought, displaying its ability to perform OA (Blum, 2017). As noted in Section 3.5.2.1 above, cultivar ranking observed for WUE (i.e. Trojan cultivar showed the highest WUE mean) seemed to indicate an indirect achievement of improved drought tolerance of modern PRG cultivars.

The deep rootedness was noticeable at harvest in HWUE genotypes of cultivar 'Trojan' (Figure 3.5C and 3.5D). Also, with the increase in RDW_D, there was a simultaneous reduction in RDW_T of elite plants (Figure 3.4C), with an increase in SMC at 30–40 cm soil depth at the end of the drought challenge (Figure 3.3B). This observation was further confirmed by the mathematical pattern captured by PC3 of PCA3.1, indicating the key trait association between WUE and OP, together with a negative PC-coefficient for RDW_T (–0.195) and a large positive coefficients for RDW_D (+0.430) and SMC (+0.383). The offset between RDW_T and RDW_D resulted in less genotypic diversity in RSR of elite genotypes in the current experiment (Figure 3.4B). For similar reasons, previous studies disregarded the importance of RSR as a selection trait for drought tolerance of temperate grasses (Bonos et al., 2004; White & Snow, 2012).

Morgan (1984) asserted that one of the major roles of OA is to maintain an average RSR through the maintenance of rooting in different soil profiles that may ensure efficient soil water extraction during drought and also post-drought regrowth (Abid

et al., 2018; Chaves, 2002; Lopes & Reynolds, 2010). It was found that there is a very low proportion of functional roots in the topmost proportion of the root system (that largely determines the magnitude of RDW_{Tot} and RSR) compared to that in the deeper root fraction of grass species under imposed drought (Bonos et al., 2004; Chaves & Oliveira, 2004; White & Snow, 2012). Therefore, deep rootedness has long been considered a useful selection trait for pasture including PRG drought tolerance (Crush et al., 2002).

3.5.3 Yield advantage of elite genotypes due to OP-WUE interplay showed little dependence on gas exchange adaptations: suggestions for further research

3.5.3.1 Leaf gas exchange-OP

It has been reported that OP and turgor driven processes (i.e. SC) are interrelated in stressed plants (Flexas et al., 2006; Ritchie et al., 1990; Turner, 2018). As noted in Section 3.5.2 above, the key role of OA is related to shoot and root hydration as LWP keeps declining in a drought. A slower rate of decline in RWC or leaf hydration enables plants to maintain the integrity of plant physiological processes during drought (Blum, 2005; Blum, 2017; Chaves, 2002; Chimenti et al., 2006) including leaf conductance and Pn (Turner, 2018). Also, more negative OP may allow further reduction in LWP without a complete turgor loss (Blum, 2017). This may induce partial stomatal closure and so reduces evaporation of water off surfaces inside the leaf for maintaining efficient leaf WU under drought (Section 2.2.4.4).

Theoretically, partial stomatal closure, which is the first plant response to occur in a drought, prevents excessive evapotranspiration but at the cost of CO_2 intake and with a negative side effect; increased T_L or VPD_L (Section 2.2.4.4). Conversely, transpiration efficiency, which is directly proportional to intrinsic WUE, is found to be increased with the partial stomatal closure (Ludlow & Muchow, 1990). With a progressively increasing drought, the potential role of OA comes in to play and has been found to be independent from gas exchange of drought-exposed plants (Blum, 2017; Blum & Tuberosa, 2018; Sinclair & Ludlow, 1986). In this context, it was salient to note that HWUE genotypes of the cultivar Trojan, that exhibited the lowest OP and the highest WUE and SDW, had average Pn rates (Section 3.5.2) despite their comparatively lower SC and ET rates (Figure 3.5). Possible explanations for this observation, as suggested by two authors, are: (1) Flexas et al. (2016) found that

under drought reduced SC may result in high intrinsic WUE even at the expense of CO_2 intake, with the involvement of mesophyll CO_2 conductance which was not recorded in this experiment and (2) Blum (2017) reported that photosynthetic products including water soluble sugars may contribute to OA that in turn supports the continuance of photosynthesis under drought.

Current results showed that 'day-of-measurement' and 'replicate' effects of Pn, SC, ET, T_L, and VPD_L were significant ($p < 0.05$), suggesting significant data noise. This may be explained by the spread of measurements throughout the day and between days that may well have contributed to the data noise observed for gas exchange data recorded in the current experiment. LI-COR measurements spanned four days and those were carried out in time windows from 10.00 am to 12 noon and 2.00 pm to 4.00 pm each day. Therefore, some effort is needed to minimise error variance of gas exchange data, if such instantaneous data are considered for face-value implications together with time-integrated measurements such as actual WUE and plant DW in future research.

3.5.3.2 Preferential dry matter partitioning and OP

The current results demonstrate that osmotically-adjusted HWUE genotypes are less dependent on net assimilation (i.e. they have low or average Pn) for production (i.e. high SDW) under imposed drought. According to Blum (2005), this trait response is comparable to a dehydration avoidance strategy, that assists plants to resist soil moisture deficit by restricting leaf water loss followed by gas exchange and net assimilation. However, the trait behaviour of elite plants observed in the current experiment was at variance with Blum's (2005) interpretation of 'dehydration avoidance' because a subset of the HWUE selection showed higher SDW and improved WUE together with better survival (i.e. high regrowth and RDW_D) compared those of the LWUE selection. Therefore, it can be speculated that OA may well have supported preferential partitioning of assimilates for the growth of young PRG tissues in the elite genotypes without compromising plant production through reduced Pn.

Turner et al. (2008) explained the importance of preferential assimilate partitioning (e.g. water soluble sugars with 3–8 degree polymerisation; fructans) for the drought tolerance of PRG genotypes. There was an apparent redirection of assimilates in the

rooting behaviour of a subset of HWUE genotypes in the current experiment, where RDW_T showed a simultaneous decrease with increase in RDW_D while RSR and LMR were unchanged (Figure 3.4). This trait behaviour may also explain how the HWUE genotypes maintained high SDW irrespective of a lesser involvement of gas exchange under imposed drought. The literature also suggests that the maintenance of an optimum RSR is one of the key roles of OA in drought-tolerant plants (Turner, 2018). Moreover, previous research has demonstrated the importance of sugar accumulation in leaf tissues for the maintenance of fundamental plant metabolic processes (Abid et al., 2018; Borrajo et al., 2018; Sallam et al., 2019; Sánchez et al., 1998; Turner et al., 2008) including protein and cell wall synthesis rather than contributing to guard cell expansion for SC under drought stress (Munns, 1988). However, the current results did not support any of these hypotheses. Therefore, a second experiment is needed to explore research questions unaddressed in the current experiment (see Chapter 4 below).

3.6 Conclusions

This experiment tested a methodology for applying a moisture deficit challenge to single potted PRG genotypes, and measuring water relations traits, including WUE. The comparison of two clonal replicates of 220 individual genotypes confirmed a large genotypic variation in WUE and associated traits within PRG cultivars evaluated under imposed drought that may underlie population improvements in modern cultivars for summer yield. A PCA (PCA3.1) established two distinctive drought-response trait associations that were indicative of high WUE in elite PRG genotypes: (1) more negative OP and increased root growth (i.e. high RDW_D and RDW_T) that promoted greater extraction of soil moisture to maintain assimilation and enhance DM production per gram of WU and (2) strongly negative OP and increased deep root growth (i.e. high RDW_D and low RDW_T) that led to SMC conservation and conferred true WUE with high SDW, apparently without the involvement of high Pn under the conditions tested. The latter response pattern is clearly of interest in breeding PRG for drought tolerance and that was the basis for performing the divergent selection (i.e. HWUE and LWUE) of the source population for WUE in this experiment. HWUE genotypes of Nui, Samson, and Trojan cultivars showed increments of 46%, 48%, and 60%, respectively, in SDW, with a significant improvement in the key traits (i.e. 1.8-fold increment in WUE) compared to those of low WUE genotypes of the three cultivars. Thus, the cultivar differences observed within the elite PRG group for WUE indicated that commercial plant

breeding has indirectly resulted in higher DM produced per gram of WU in modern cultivars (i.e. Trojan) than older cultivars (i.e. Nui) without a specific focus on the WUE trait. Therefore, current results suggest that the identified traits may effectively be used in selecting PRG for drought tolerance, if the consistency of this conceptually ideal pattern of trait associations involving true WUE is established.

Chapter 4

Retesting of LWUE and HWUE selections for the consistency of the key trait expression across two growing seasons with a further trait evaluation in a soil moisture deficit of an extended duration

4.1 Abstract

This chapter describes Experiment 2 that retested LWUE and HWUE plants selected from the previous experiment (summer 2017/18) in a second growing season (summer 2018/19). Two clonal ramets of 20 HWUE genotypes (Nui, n=5; Samson, n=7; Trojan, n=8) and 15 LWUE genotypes Nui, n=7; Samson, n=5; Trojan, n=3) divergently selected in the first experiment were grown in 55 cm tall pots and after three months of root development near field capacity (FC), were exposed to a 2-month period of progressively increasing water deficit. Nine water relations traits as in Experiment 1 (i.e. SDW, RDW, RDW_T, RDW_D, WUE, OP, LWP, RWC, SMC_D, and post-cutting regrowth) were first evaluated to ascertain the consistency of expression of the key trait associations in both selections. Additional morphological trait measurements (i.e. tiller number, TN; tiller appearance rate, TAR; leaf elongation rate, LER), biochemical characteristics (i.e. low and high molecular weight water soluble carbohydrates (LMWWSC and HMWWSC, respectively) and proline concentration), selected photosynthetic gas exchange measurements (i.e. SC and Pn), and chlorophyll fluorescence parameters (i.e. Fv/Fm, F'v/F'm, φPSII, qP, NPQ, and ETR) were performed to evaluate the role of these other traits in drought-response patterns as identified in three measurement cycles at progressively decreasing soil moisture levels (75–85% FC, 55–65% FC, and 35–45% FC). Water-use efficiencies averaged for the HWUE and LWUE groups were 567±16 g WU/g SDW and 918±44 g WU/g SDW, respectively, with 46%, 68%, and 78% increments in HWUE genotypes of Nui, Samson, and Trojan cultivars, respectively, compared to LWUE genotypes of three cultivars. Mean SDWs of HWUE and LWUE groups were 6.4±0.23 and 4.5±0.36 g/ plant, respectively. Moreover, HWUE genotypes exhibited more negative OP and higher RWC at low LWP (i.e. –1.0 MPa) than the LWUE genotypes together with higher RDW_D, regrowth, and a reduced rate of decline in SMC. For OP, SDW, RDW_D and WUE correlations for key traits across seasons were, respectively, 0.910, 0.878, 0.811 and 0.756 ($p < 0.001$), with only the

RDW_T cross-season correlation not significant at p = 0.05. PCA 3.1 that originally included data for nine traits from 220 genotypes in Experiment 1 was recalculated for the same nine traits using data for only the 35 divergently-selected HWUE and LWUE genotypes (PCA 3.3). After recalculation, PC3 of PCA 3.1 was promoted to PC1 in PCA 3.3, as judged by trait coefficient patterns in PCA3.3. Also, Experiment 2 data for the same 35 HWUE and LWUE genotypes for the same nine traits reassessed by another PCA (PCA 4.1) and PCAs 3.3 and 4.1 were compared to test the consistency of drought-response patterns across experiments. It was seen that trait associations for Experiment 1 identified by PCs 1 to 3 of PCA 3.3 were similar to those for Experiment 2 identified by PCA 4.1, as judged by cross-correlation of PC scores in PCAs 3.3 and 4.1 for the 35 genotypes. In particular, PC1 in PCA 3.3 (that aligned with PC3 in PCA 3.1) and PC1 in PCA 4.1 defined a similar SDW-WUE-OP-RDW_D trait association, with SMC_D-conservation in both cases. Inclusion of data for additional traits not measured in Experiment 1 in PCA 4.1 (PCA 4.2) again affected the order of trait associations across PCs, but revealed that both the PCA 4.1-PC1 'SMC_D-conserving' trait association and the PCA 4.1-PC3 'SMC_D-depleting' trait association involved higher levels of shoot HMWWSC, while Pn, SC and Fv/Fm were negatively impacted in the SMC_D-conserving trait association and enhanced in the SMC_D-depleting trait association. These findings support the characterization of the SMC_D-conserving trait association as 'true WUE', and the characterization of the SMC_D-depleting trait association as 'effective use of water'. The current results engender confidence that the drought tolerance traits identified in the subset of plants divergently selected in Experiment 1 based on PC3 scores of PCA3.1 are consistently expressed across seasons, meeting the first requirement for use as selection criteria in screening PRG germplasm for drought tolerance.

4.2 Introduction

Experiment 1 in summer of 2017/2018 used two replicates of 50, 80, and 90 PRG genotypes each, of cultivars Nui, Request, and Trojan, respectively, tested a methodology to provide a moisture deficit challenge to single PRG genotypes over a period of about a month, and measured the water consumption and yield during water deficit (Chapter 3). Results of Experiment 1 identified a WUE trait response pattern of interest (PC3 in PCA3.1; Table 3.3). Accordingly, two contrasting groups of PRG genotypes were selected from the three cultivars (twenty 'HWUE genotypes' and fifteen 'LWUE genotypes') where the group of HWUE genotypes was characterised by high SDW, improved WUE (with a two-fold increment in HWUE selection compared to the LWUE selection for WUE), more negative OP, deep rootedness, and high post-cutting regrowth. Moreover, there was a good agreement between clonal replicates for the key traits and was considered to provide a sound basis for selection of elite PRG genotypes with enhanced drought tolerance for future research or breeding, if the traits of interest are consistently expressed or heritable. Literature suggests that the consistency of the trait behavior across growing seasons is of the utmost importance when selecting plants for drought tolerance in any breeding program (de Almeida Silva et al., 2012; Kemp & Culvenor, 1994; Wedderburn et al., 2010). Also, it is widely held that the performance of agronomic selection traits should be validated at least in two independent experiments (Kemp & Culvenor, 1994).

Given the importance of evaluating the consistency of results in selection experiments, the main objective of this experiment was set to test the repeatability of the trait performance of the selected PRG genotypes from Experiment 1 in a second growing season (2018/19 summer). Hence, Experiment 2 involved the same set of trait data (i.e. nine water relations traits including WUE, SDW, OP, LWP, RWC, RDW_T, RDW_D, SMC_D, and RGS) together with additional traits that are likely to be linked to the major traits of interest based on the questions arising from the results of Experiment 1 (Section 3.5). The aim in Experiment 2 was for plant expression of these traits to be evaluated at three points across a drying cycle longer than that in Experiment 1, but including collection of data at a similar soil moisture deficit to that in Experiment 1 for the comparison of results across the two experiments.

This chapter provides details including methodology, results and discussion and, conclusions of Experiment 2 conducted in the summer 2018/19. The results section first overviews the trait behavior of HWUE and LWUE selections (twenty and fifteen genotypes, respectively) for the nine water relations traits under moderate drought as imposed in Experiment 1 and secondly, for all measured traits across different soil moisture phases imposed throughout Experiment 2. Then, the key drought-response trait association of test genotypes is further evaluated in two sub-sections; the first sub-section explores the association between more negative OP, high WUE, and associated secondary traits and the second sub-section assesses the contribution of gas exchange and chlorophyll fluorescence parameters to the key trait association and PRG drought tolerance of test genotypes under the imposed drought phases. Finally, the trait expression and drought-response patterns of those traits of the HWUE and LWUE selections that were measured in both experiments are compared in order to establish the consistency of the key trait performance across the two growing seasons. Corresponding discussion on these sub-sections is then provided in Sections 4.5.1 and 4.5.2.

4.3 Materials and Methods

4.3.1 Plant material

Clonal copies of the 20 low WU genotypes (i.e. HWUE; Nui, n=5; Samson, n=7; Trojan, n=8), and 15 high WU genotypes (i.e. LWUE; Nui, n=7; Samson, n=5; Trojan, n=3) selected from Experiment 1 (Chapter 3.3) were established in new pots to provide the plants for use in Experiment 2. Similar-sized clonal ramets (4–5 tillers each) of the selected PRG genotypes were sourced from stock plants that were established in June 2017 from seeds carrying the AR1 endophyte, and maintained indoors with regular trimming, fertilizer application, and repotting as required.

4.3.2 Experimental set up

This study was conducted as a pot experiment in a glasshouse environment at the Plant Growth Unit, Massey University, New Zealand (40.3709° S, 175.6303° E, 35 m.a.s.l.) during summer, from June 2018 to January 2019. Pots (sections of PVC water pipe 50 cm tall and 10 cm in diameter, with an end cap made from a double thickness of nylon horticultural shade mesh) were lined with tubular polythene

plastic for easy root harvesting in the end (with perforations at the bottom to allow drainage and aeration) and a saucer kept at the bottom of each pot to capture excess drainage if there was any. Pots were filled with fertilised 'A' horizon of an Egmont Black Loam soil with WHC >65%, measured as MW/MS. Pots were packed to a standard weight of 3.7–3.9 kg of air-dried soil per pot (MW/MS approximately 5% and bulk density 1.1–1.3 g/cm^{3}in each pot) similar to that in Experiment 1.

Two clonal replicates of the 35 genotypes from the two selections to be tested were transplanted into experimental pots and arranged in a row-column design (i.e. randomised complete block design) in the glasshouse (Figure 4.1). In here, both sets of experimental plants from this Experiment 2 and Experiment 3 (i.e. two types of commercial breeder's material; see Chapter 5 below) were inter-randomized (with different colour tags for easy identification) within the same glasshouse space (Figure 4.1 and 4.A1) to obtain common trait measurements for both experiments for further data analyses in Chapter 5 below.

Pots were first maintained at near FC for eight weeks to allow tillering to ≥20 tillers/pot and to ensure good root development. Genotypes were then defoliated to 5 cm above the soil surface and the subsequent 'measurement' regrowth was allowed.

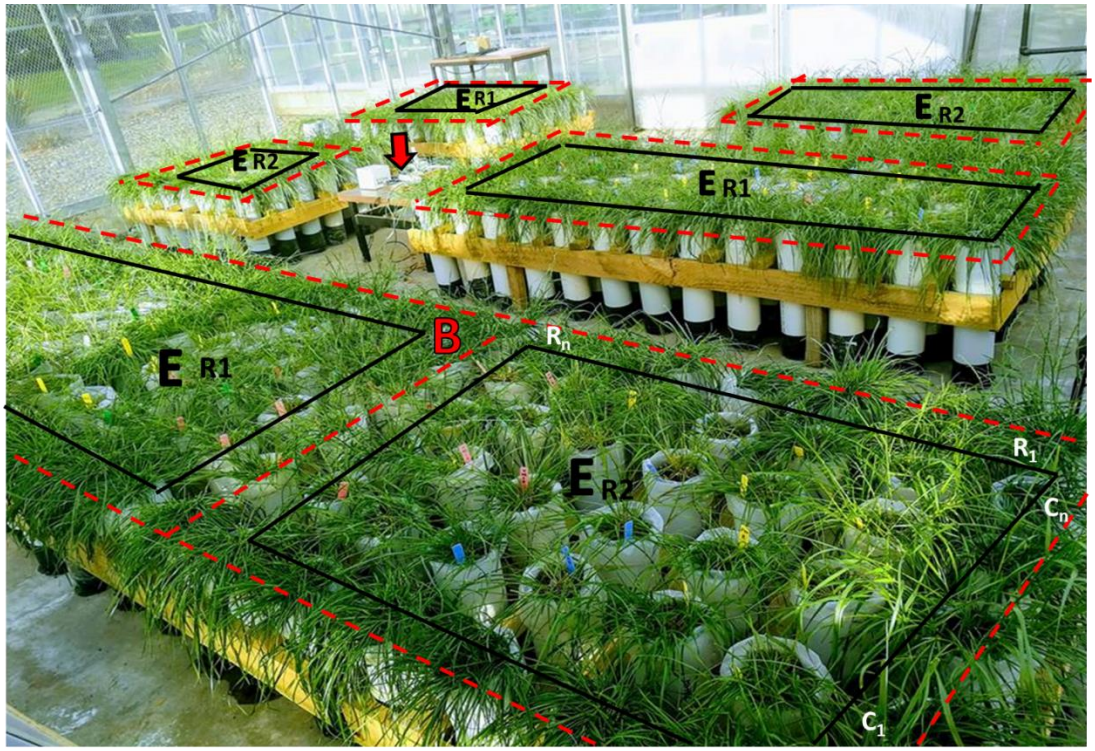

Figure 4.1 The general view of how the experimental pots (E) of Experiment 2 and 3 were inter-randomized within the same glasshouse space (with different colour tags (see also Figure 4.A1)) and with border plants (B) (Note: 'R1' and 'R2' denote replicates 1 and 2, respectively; R_1…R_n, Rows; C_1….C_n, Columns; 'E' represents single-potted clonal replicates of perennial ryegrass genotypes from the high- and low WU selection from Experiment 1 that were retested in Experiment 2 and subsets of genotypes from two commercial breeding lines that were tested in Experiment 3 in comparison to the low WU selection of Experiment 2; The arrow in the figure indicates the light and temperature data collection unit located at the canopy level inside the glasshouse).

4.3.3 Drought treatments

The mean maximum and minimum glasshouse daily temperatures were 28°C and 15°C, respectively. The relative humidity was typically within the range 55–85%, with light intensity between 800–1000 µmol photons/m^2/s at the canopy level (as measured by the light and temperature data collection unit (Skye Datahog fitted with PAR and temperature sensors reading sensors every 20 minutes and recording hourly means) located at the canopy level inside the glasshouse; see Figure 4.1) at midday. Moisture deficit was imposed from mid-November 2018, starting with pot

soil moisture contents at FC after root development near FC for two months, and then reducing irrigation interval and volume progressively, allowing pots to reach mild drought (70–80% FC or MW/MS ~ 50% maintained for 15 days), moderate drought (55–65% FC or MW/MS ~ 40% maintained for 34 days) and, intense drought (35–45% FC or MW/MS ~ 30% maintained for another 34 days) giving a total experiment duration of ten weeks (Figure 4.2). The totals of plant WU and soil moisture status were determined by weighing pots individually on an electronic balance to 1 g precision at each defoliation stage (i.e. end of each soil moisture phase). As noted in Chapter 3.3.3, samples of the soil used for filling pots, when tested on a pressure plate apparatus at –0.01, –0.1, and –1.5 MPa had gravimetric soil moisture of 66, 46, and 36%, respectively. Regular soil moisture measurements were recorded by using a time domain reflectometer (TDR) at two soil depths (10–20 cm and 40–50 cm) (Figure 4.2). As an alternative indicator, plant stress levels at mild, moderate, and intense drought conditions were recorded by measuring LWP of randomly selected PRG genotypes of the three tested cultivars. The recorded values were –0.6 MPa, –0.8 MPa, and –1.0 MPa, respectively. Ten weeks after the first reduction of water supply when genotypes had completed a period of intense drought or M2 (55−65% FC) (i.e. after the last defoliation; M4 stage as presented in Figure 4.2 below), irrigation was completely withdrawn and regrowth was allowed for another week for visual scoring of regrowth. Detailed measurements including yield, key plant water relations, and associated traits were carried out. To facilitate these trait measurements, samples were collected three to five times over a two-day time window in each moisture deficit phase during the experiment as set out in the schedule below (Figure 4.2) and after collection dried or frozen for further analysis as set out in Section 3.4.

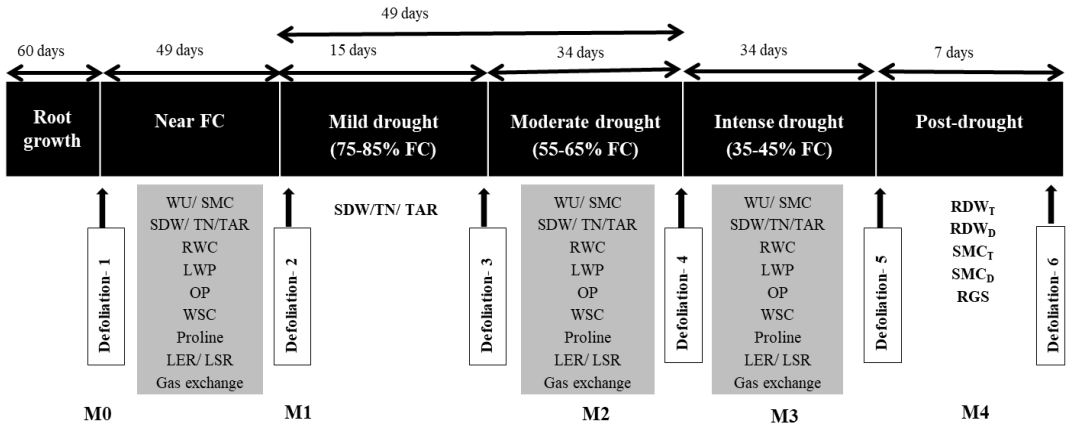

Figure 4.2 Treatment and data collection schedule of Experiment 2 (Trait abbreviations: FC, Field capacity; WU, Pot water use; SDW, Shoot dry weight; TN, Tiller number; TAR, Tiller appearance rate; RWC, Leaf relative water content; LWP, Predawn leaf water potential; OP, Leaf osmotic potential; WSC, Water soluble carbohydrates; LER, Leaf elongation rate; LSR: Leaf senescence rate; RDW_T, Root dry weight at 4–20 cm depth; RDW_D; Root dry weight at 20–50 cm depth; SMC_T gravimetric soil moisture content at 10–20 cm depth; SMC_D gravimetric soil moisture content at 40–50 cm depth; RGS, Post–cutting regrowth score) Note: gas exchange measurements included photosynthesis or stomatal conductance and chlorophyll fluorescence parameters depending on the drought phase in which the measurements were made and M0, M1, M2, M3, and M4 represent defoliation and measurement periods near FC and in mild drought, moderate drought, intense drought, and post-drought phases, respectively.

4.3.4 Measurements and analyses

4.3.4.1 Plant-water relations traits

Shoot samples were collected by clipping to 5 cm above the soil surface (the same height as at the first cutting) at the end of each drought phase. Tiller appearance, leaf elongation (LER) and/or, leaf senescence rates (LSR) were recorded every five days over a three week time window during each drought phase, following defoliation. A sub-set of ‘adult’ tillers (i.e. two to three tillers per plant) were randomly selected and tagged with coloured plastic rings for regular LER and/ or LSR measurements. Cumulative leaf length measurements were taken by measuring three to five green leaves, from the top of each emerging leaf lamina to the ligule of the next oldest

leaf, of each selected tiller (Hatier et al., 2014; Horst et al., 1978). Roots were harvested once at two different depths (as described in Section 3.3.4 above) in the end of the experiment (i.e. RDW_T, 10–20 cm; RDW_D, 20–50 cm). Both shoot and cleaned root samples were oven-dried separately at 80°C for 48 hours, and dry weights were recorded. Root to shoot ratio (RSR %) was calculated as a percentage ratio between the total RDW and the total SDW (including crown) and the proportion of deeper roots was also calculated. Watering was completely withdrawn at the end of the intense drought and then test genotypes were defoliated leaving 5 cm of stubble. Post-cutting regrowth was visually scored 7 days after defoliation on a 0 to 5 scale, based on live tiller number as a percentage of the total tiller number (tiller bases) as describes in Section 3.4 above.

Pot WU was recorded throughout and WUE (g H_2O/g DM) was calculated using pot WU and oven-dried clipped shoot dry weight data at each drought phase in addition to regular soil moisture measurements. Post-drought SMC was calculated using destructively collected soil samples at two depths (i.e. SMC_T at 10–20 cm and SMC_D at 40–50 cm) for each pot in the end of the experiment. The SMC (%, w/w) was calculated as SMC (%, w/w) = (WW – DW)/DW × 100%, where WW and DM denote wet weight and dry weight of the soil samples, respectively. Soil dry weight was taken by oven-drying approximately 300 g of soil at 105°C for 48 hours.

Predawn leaf water potential, OP, and RWC of the youngest fully expanded leaf of representative tillers of each individual genotype were measured as described in Chapter 3.3.4 above. Representative samples of growing tillers, including expanding leaf blades, mature leaves, the main tiller pseudostem, and associated leaf sheaths were collected before defoliation from each genotype at each drought phase, immediately frozen in liquid nitrogen and, stored at –80°C, for analysis of proline and water soluble carbohydrates (Section 4.3.4.2 below).

4.3.4.2 Biochemical parameters

4.3.4.2.1 Water soluble carbohydrate (WSC) analysis

For the quantification of both low molecular weight (LMWWSC) and high molecular weight (HMWWSC) water soluble carbohydrates or sugars, representative leaf samples previously frozen at –80°C (4.3.4.1 above) were freeze-

dried for three days and ground to powder using a Retsch MM200 mixer mill. A sub sample of approximately 25 mg of homogeneously ground material from the two replicates of each genotype was subjected to sugar extraction as described by (Pollock & Jones, 1979), followed by anthrone analysis as originally described by (Jermyn, 1956), and then employed by other researchers (Liu et al., 2020; Parsons et al., 2004; Rasmussen et al., 2009; Smith et al., 2002; Tang et al., 2013; Yu et al., 2012) in PRG research.

(A) Extraction of LMW sugars

Approximately 25 mg freeze-dried and finely ground leaf tissue was weighed into 2 ml Eppendorf tubes and 1 ml of 80% ethanol (v/v) was added to each. Samples were vortexed for 10 seconds, shaken in a hot water bath for 30 minutes at 65ºC and then centrifuged for 15 minutes at 13000 rpm. Supernatant was pipetted into another 2 ml Eppendorf tube and kept aside, and then 1 ml of 80% ethanol (v/v) was added to the residue. The residue containing ethanol was vortexed for 10 seconds, shaken in a hot water bath for 30 minutes at 65ºC and, centrifuged for 15 minutes at 13000 rpm. The supernatant was pipetted and combined with the previous supernatant and stored at –20ºC for analysis (Note: The green coloured extract was used for the LMWWSC analysis without clearing pigments). This procedure was understood to test for glucose, fructose, sucrose, and small oligomers including LMW fructans (i.e. degree of polymerization less than 3) (Pollock & Jones, 1979).

(B) Extraction of HMW sugar polymers

Assuming that PRG extracts contain negligible amounts of starch, HMW fructans (i.e. degree of polymerization 3–8) was expected to be the main constituent detected in the HMWWSC fraction remaining after the above extraction of LMW sugars. To quantify this fraction, 1 ml of H2O was added to the residue from LMW analysis, was vortexed for 10 seconds, shaken in a hot water bath for 30 minutes at 65ºC and then, the mixture was centrifuged for 15 minutes at 13000 rpm. Supernatant was pipetted into a 2 ml Eppendorf tube and kept aside and 1 ml of H_2O was added to the residue. The residue containing H_2O was vortexed for 10 seconds, shaken in a hot water bath for 30 minutes at 65ºC and then centrifuged for 15 minutes at 13000 rpm. Supernatant was pipetted and combined with the previous supernatant and stored at –20ºC for analysis.

(C) Anthrone analysis

Anthrone reagent was freshly prepared each day analyses were performed, using 100 mg of anthrone. Thirty milliliters of 100% ethanol was cooled in ice and then 50 ml of conc. H_2SO_4 was added slowly to the ethanol inside a fume hood. The mixed solution was cooled down to room temperature and the anthrone was mixed homogeneously.

For LMW, 12 µl extracts were taken from each sample into microwells and 188 µl of H_2O was added to make a master diluted sample. The master sample was mixed thoroughly by careful pipetting. Fifty microliters was removed from the diluted master sample to a new microwell and 250 µl of Anthrone reagent was added to that. Three replicates were made for each sample. Standards were prepared using sucrose at concentrations of 0, 10, 20, 30, 40, 50, 75, and 100 µg/ ml. Microwells containing triplicate samples and standards were incubated at 65ºC for 25 minutes (Note: the oven was warmed to 65ºC one hour prior to incubation). Absorbance of samples was read at 620 nm using a spectrophotometer (Wallac 1420 Micropplate Reader Perkin Elmer®, GMI, USA).

For HMW, 40 µl extracts were taken from each sample into microwells and 160 µl of H_2O was added to make the master diluted sample. Master samples were mixed thoroughly by careful pipetting. Fifty microliters was removed from each diluted master sample to a new microwell and 250 µl of anthrone reagent was added to it. Three replicates were made for each sample. Standards were prepared using inulin at concentrations of 0, 10, 20, 30, 40, 50, 75, and 100 µg/ ml. As for LMW, microwells containing triplicates of samples and standards were incubated at 65ºC for 25 minutes in an oven pre-warmed to 65ºC for one hour prior to incubation, and then absorbance was read at 620 nm as above.

4.3.4.2.2 Proline analysis

A simplified rapid colorimetric method was used for proline analysis. For quantifying free proline, shoot samples previously frozen at –80°C were freeze-dried and ground to powder using a Retsch MM200 mixer mill as described above. Sub samples of approximately 50 mg of powdered plant tissue from two replicates of each genotype were then subjected to the proline analysis (Bates et al., 1973; Magné

& Larher, 1992). Ninhydrin reagent was freshly prepared using 1% ninhydrin (w/v) in 60% glacial acetic acid (v/v) for each batch of analyses. Standards were prepared from a ready-made stock solution of free proline (600 µg/ml) and stored at 4°C.

Fifty milligrams of each sample was weighed in to a 1.5 ml Eppendorf tube and was suspended in 1.2 ml of 3% (w/v) sulphosalicylic acid to precipitate protein. The solution was then vortexed for 10 seconds and centrifuged at 3000 rpm for 10 minutes at room temperature. The resulting supernatant was transferred into a fresh 1.5 ml Eppendorf tube. A combined solution was prepared using a 200 µl aliquot of supernatant, 400 µl of water and, 800 µl of 1% ninhydrin reagent. Samples were then incubated in a water bath for 1 hour at 98ºC. The reaction was stopped by plunging samples into ice and returning them to room temperature. Protein-ninhydrin chromophore product was then extracted in 800 µl of toluene in a fume hood. Extracts were vortexed for 15–20 seconds and samples were left to stand for around 15 minutes for phase separation to occur. The upper toluene phase was transferred to a 1 ml quartz cuvette for spectrophotometric analysis against a toluene blank. Sample absorbance of each sample was compared to a set of separately prepared proline standards (0, 5, 10, 15, 20 and 30 µg/ml) using a spectrophotometer (Novaspec® III+ Spectrophotometer, Amersham Biosciences, UK) at 518 nm in a fume hood. The concentration of free proline for each sample was adjusted for initial tissue dry weight (Bates et al., 1973; Magné & Larher, 1992).

4.3.4.3 Gas exchange & chlorophyll fluorescence measurements

Instantaneous gas exchange variables including net photosynthetic rate (Pn) and stomatal conductance (SC) were measured in a fully-expanded youngest leaf of two representative tillers of each genotype under artificial, saturating photon flux density (1000 µmol/m/s) at an ambient CO_2 concentration of 400 ppm using a portable photosynthesis meter (LICOR 6400XT, LICOR Biosciences Inc., NE, USA). Measurements were performed for the fully-expanded youngest leaf from two representative tillers of two replicates of each genotype from 10.00 am to 12.00 noon for three consecutive sunny days nearing the end of each drought phase. Gas exchange data were recalculated according to the actual total leaf area fitted into the circle shape leaf chamber (diameter: 3 cm): $\sum^{n=2}$(3 cm × (leaf width)). The temperature of the leaf chamber during measurements was around 27°C. The

relative humidity (RH) of the chamber was controlled within a range of 55–65%, similar to daytime RH in the glasshouse.

Chlorophyll fluorescence parameters were recorded once, in the last week of the intense drought phase or at M2 (55−65% FC) according to recommended procedures given in the LICOR 6400XT manual (LICOR Biosciences, Inc., Lincoln, NE, USA). Measurements were taken in both dark-adapted leaves (i.e. overnight dark adaptation followed by predawn measurements) and the same leaves in a light-adapted state later in the day (i.e. dark-adapted leaves were exposed to light after morning measurement and measurements were taken from 10.00 am to 12.00 noon on the same day) using the same fully expanded youngest leaf of two randomly chosen adult tillers of each plant. Measurements continued for two consecutive days in order to measure all genotypes. An open gas exchange system (LICOR -6400XT; LICOR Biosciences, Inc., Lincoln, NE, USA) was used for these measurements with an integrated leaf fluorescence chamber (LICOR-6400-40 leaf chamber-fluorometer; LICOR Biosciences, Inc., Lincoln, NE, USA). Predawn measurements were recorded as (1) minimum fluorescence (Fo) and (2) the maximum fluorescence (Fm) and the maximum quantum efficiency of photosystem II (PSII) was calculated as: Fv/Fm = (Fm − Fo)/Fm. Similar measurements were performed for light-adapted leaves and the energy harvesting efficiency of PSII was calculated as: F'v/F'm =(F'm − F'o)/F'm, where F'o is the minimal fluorescence in light-adapted state and F'm is the maximum fluorescence value. The estimate of the relative quantum yield of photosystem II (φPSII) was calculated as: φPSII = (F'm − Fs)/F'm using the steady-state parameter (Fs). Photochemical quenching (qP) and non-photochemical quenching (NPQ or qN) were calculated as: qP = (F'm − Fs)/(F'm − F'o) and NPQ or qN = (Fs/F'm) − (Fs/Fm), respectively (Hendrickson et al., 2004). The electron transport rate (ETR) was calculated as: ETR = PPFD × φPSII × 0.85 ×0.5 (Assumptions: leaf absorption is 0.85 and PSII: PSI ratio is 1:1).

4.3.5 Statistical analyses

Data from two replicates of 20 HWUE and 15 LWUE genotypes for each trait were subjected to ANOVA using Proc GLM in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) to examine the WUE group means, cultivar means within groups, and statistical probabilities for group and cultivar effects of measured traits under

imposed drought. It was assumed that data were normally-distributed and there was no data transformation required to meet ANOVA assumptions. Comparisons between means were made using the LSD mean separation subcommand for relevant figures and tables.

Averaged data from the two replicates of 35 HWUE and LWUE genotypes were subjected to two PCAs in Minitab version 14 to explore key drought-response patterns of selected groups in Experiment 2 in two steps. Firstly, a PCA (PCA 4.1) was performed for the nine water relations traits that were initially analysed in Experiment 1 and then reanalysed in Experiment 2 using the same data input structure as in Experiment 1. Next, a second PCA (PCA 4.2) was performed for all 23 traits that were measured during the intense drought phase or during M2 (55−65% FC) in Experiment 2, including morpho-physiological, biochemical, gas exchange, and CF trait measurements, for detailed interpretation of the trait data. An additional PCA (PCA 3.3) was performed using the nine key water relations trait data of the 20 HWUE and 15 LWUE genotypes from Experiment 1 (SDW, WUE, LWP, OP, RWC SMC_D, RDW_D, RDW_T, and RGS) in order to compare that with the PCA4.1 for the consistency of trait associations and data patterns between Experiment 1 (2017/18) and Experiment 2 (2018/19) in a common PCA structure. For further comparison of data patterns for the 35 divergently selected genotypes across seasons, correlations between the two sets of 35 PC scores from PCA 3.3 and PCA 4.1 were examined using Pearson's correlation analysis.

A paired sample t-test was also performed to compare trait means between WUE groups (i.e. HWUE and LWUE) and cultivar means within groups (i.e. 'Nui', 'Samson', and 'Trojan' cultivars), to test the consistency of the results across Experiments 1 and 2.

4.4 Results

4.4.1 Consistency of trait expression between Experiments 1 and 2 for HWUE and LWUE genotypes

For PCA 4.1, of the nine available PCs, four PCs explaining 92% of the total data variation were selected for presentation (Table 4.1). PC1 explained 68.2% of the total variance, with PCs 2, 3, and 4 explaining 13.0%, 6.1% and 4.2%, respectively.

PC1 captured a pattern of trait association in individual genotypes reflecting (as indicated by PC coefficients) high SDW, RDW_D, and RGS coupled with high SMC_D (i.e. soil moisture at depth was conserved by the genotypes with high scores in PC1) and negative coefficients for g WU/g DM (indicating high WUE), and with more negative OP and LWP and reduced RDW_T (Table 4.1). PC2 identified genotypes with high SDW at higher WU and depleted soil moisture that do not show more negative OP as indicated in PC1. PC3 identified plants with a similar set of trait associations to PC1, but with stronger exploitation of deep soil water (negative coefficient for SMC_D) and attainment of higher SDW than PC1 plants as indicated by SDW coefficients of +0.533 for PC3 and +0.306 for PC1. Also, PC3 was represented by plants with lower RDW_T (negative PC co-efficient) compared to that of PC1 (positive PC coefficient) of PCA4.1 (Table 4.1). PC4 indicated an obscure trait association featuring more negative LWP (PC coefficient –0.723) coupled with low RDW_D and RGS (PC coefficients –0.483 and –0.259, respectively) and to a lesser extent lower SDW (PC coefficient –0.140) and, paradoxically, greater exploitation of SMC_D (PC coefficient –0.357).

For PCA 4.2 conducted to evaluate the additional trait measurements introduced in Experiment 2 to gain further insight into the drought-response patterns identified in Chapter 3 and in PCA 4.1, the first five PCs (66.7% of the total data variation explained and with eigenvalues >0.7), were considered biologically and statistically significant and were therefore selected for presentation (Table 4.1) from the total of twenty three PCs generated in PCA 4.2. PC1 explained 24.8% of the total variance with PCs 2, 3, 4 and 5 explaining 13.6%, 10.4%, 9.7% and 8.1%, respectively. Coefficients of the nine water relations traits in PC1 of PCA4.2 had a superficially similar structure to those of PC1 in PCA4.1 but coefficients for RDW_T and RGS had changed signs and the coefficient for SMC_D was near zero. PC1 of PCA 4.2, in addition to the modified association of water relations traits upon the addition of new traits as described above, featured positive coefficients for HMWWSC, gas exchange (Pn and SC), CF parameters except for NPQ (Fv/Fm, F'v/F'm, φPSII, qP, and ETR) and low SMC, RGS, and NPQ. PC2 of PCA 4.2 was characterised by a trait association of high LER only modestly coupled with SDW, high WU (i.e. low WUE) and depletion of SMC_D, with unfavourable PC coefficients for OP and WSC (i.e. a positive PC coefficient for OP and negative coefficients for HMW and LMW

WSC). PC2 was also independent from gas exchange and CF parameters (Table 4.1). PC3, that explained 10.4% of the total variation, described variation between genotypes indicating a similar drought-response pattern to that elucidated by PC1 of PCA 4.1, but with more positive coefficients for TAR, LER, and HMWWSC and negative or variable coefficients for proline and LMWWSC, and negative coefficients for most gas exchange and CF parameters. PC4 and PC5 were expressed as the variation among genotypes for plant hydration (i.e. hydrated and dehydrated plants, exhibiting high RWC with low proline and low RWC with high proline, respectively) and low LMW WSC in both cases (Table 4.1). The modified PC structures and trait associations arising with the introduction of 14 additional traits were statistically explored by analyzing cross-correlations of PC scores of the first five PCs between PCA 4.1 and PCA 4.2 (Table 4.2). This analysis showed that PC1 of PCA4.1 was most highly correlated with PC3 of PCA4.2 ($r = 0.973$, $p < 0.0001$), while PC3 of PCA 4.1 was most highly correlated with PC1 of PCA 4.2 ($r = 0.702$, $p < 0.0001$). However, some information migrated between PCs with the introduction of the additional 14 traits. For example, PC1 of PCA 4.2 contained information from PC1 of PCA 4.1 ($r = 0.505$, $p = 0.017$) in addition to the information from PCA 4.1 PC3, and both PC2 and PC4 of PCA 4.2 gained some information from PC3 of PCA 4.1 ($r = 0.273$ and 0.256, respectively, at $p < 0.05$). PC3, PC4 and PC5 of PCA 4.1 were not significantly correlated with the corresponding PCs of PCA 4.2 ($p > 0.05$; Table 4.2).

Table 4.1 Principal component coefficients of the first four PCs of PCA4.1 and the first 5 PCs of PCA4.2. PCA4.1 includes the nine water relations traits as evaluated in PCA 3.1, while PCA4.2 includes data for all 23 traits measured in Experiment 2. The data are for the 35 selected HWUE and LWUE genotypes measured in Experiment 2 at M2 (55–65% FC) in PCA4.1 and at M3 (45–55% FC) in PCA4.2. Coefficients less than 0.100 are suppressed.

	PCA 4.1				PCA 4.2				
	PC1	**PC2**	**PC3**	**PC4**	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**
Eigen value	6.14	1.17	0.55	0.38	15.33	2.60	1.62	0.97	0.70
%variation explained	68.2	13.0	6.1	4.2	24.8	13.6	10.4	9.7	8.1
%Cumulative variance	68.2	81.2	87.3	91.6	24.8	38.4	48.8	58.6	66.6
SDW	0.306	0.349	0.533	−0.140	0.192	0.164	0.226	0.310	0.169
WUE	−0.388	0.330	−0.223	–	−0.209	0.211	−0.161	0.171	–
LWP	−0.307	0.370	−0.143	−0.723	−0.244	−0.113	–	0.310	−0.115
OP	−0.375	0.124	−0.258	–	−0.216	0.195	−0.454	–	−0.105
RWC	0.325	−0.196	0.572	0.170	0.185	0.117	0.208	0.375	−0.243
SMC_D	0.143	−0.799	−0.307	−0.357	–	−0.438	0.405	–	−0.103
RDW_D	0.355	−0.137	0.345	−0.483	0.243	–	0.155	–	–
SMC_T					−0.166	0.246	0.304	0.109	–
RDW_T	−0.348	–	0.190	–	0.201	–	−0.363	−0.141	0.115
RGS	0.385	−0.168	–	−0.259	−0.240	–	0.100	–	–
TAR					0.211	0.291	0.116	0.114	–
LER					0.134	0.450	0.165	–	−0.282
Proline					–	−0.340	–	−0.491	0.424
LMW WSC					–	−0.289	–	−0.564	−0.752
HMW WSC					0.200	−0.276	0.261	−0.119	–
Pn					0.244	–	−0.209	–	–
SC					0.238	–	−0.269	–	–
Fv/ Fm					0.234	–	−0.275	–	–
Fv'/ F'm					0.242	–	−0.215	–	–
The efficiency of PSII					0.249	–	−0.152	–	–
Photochemical quenching					0.245	–	−0.148	–	–
Non-photochemical quenching					−0.248	–	0.100	–	–
Electron transport rate					0.243	–	−0.230	0.148	0.148

Trait abbreviations: SDW, Shoot dry weight; TAR, Tiller appearance rate; LER, Leaf elongation rate; WUE, Water–use efficiency; LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC_T and RDW_T, Gravimetric soil moisture content and root dry weight at 10–20 cm depth; SMC_D and RDW_D, SMC and RDW at 20–50 cm depth; RGS, Post-cutting regrowth score; LMWWSC and HMWWC, Low and high molecular weight water soluble carbohydrates; Pn, Photosynthesis; SC, Stomatal conductance; Fv/ Fm and F'v/ F'm, The maximum photochemical efficiency of PSII of dark-adapted leaves and light-adapted leaves.

Table 4.2 Cross-correlations between PCA4.1 and PCA4.2 PC scores for the first five PCs of each PCA. PCA4.1 includes nine key water relations traits measured at M2 (55−65% FC) while PCA4.2 includes 23 morpho-physiological traits of the 35 divergently selected genotypes measured at and M3 (45−55% FC), in Experiment 2

	PCA4.1				
	PC1	**PC2**	**PC3**	**PC4**	**PC5**
PCA4.2					
PC1	0.505[R]	0.202	0.702	0.028	−0.052
	0.017[P]	0.245	0.000	0.871	0.768
PC2	0.173	0.838	0.273	0.050	−0.052
	0.321	0.000	0.041	0.774	0.767
PC3	0.973	−0.400	−0.060	0.048	−0.108
	0.000	0.017	0.731	0.785	0.537
PC4	0.043	0.139	0.256	−0.091	−0.318
	0.807	0.426	0.038	0.605	0.063
PC5	0.032	0.180	0.223	0.262	−0.008
	0.857	0.302	0.198	0.029	0.965

(Note: [R]=Pearson correlation coefficient; [P]=p-value; It is seen that PC1 of PCA4.1 was most highly correlated with PC3 of PCA4.2, while PC3 of PCA 4.1 was most highly correlated with PC1 of PCA 4.2).

In addition to PCAs 3.1 and 3.2 presented in Section 3.4 above for the 220 PRG genotypes, studied in Experiment 1, water relations trait data of the 35 selected HWUE and LWUE genotypes were extracted from the Experiment 1 data and a new PCA (PCA3.3, Table A4.1) was performed. Cross-correlations of PC scores of the first five PCs of PCA4.1 and PCA3.3 were then calculated to evaluate the consistency of the key trait performance across two experiments (Table A4.2). It was found that all five PCs of PCA4.1 were highly correlated with those of PCA3.3 ($r \geq 0.50$, $p < 0.05$) even though some information from PC2, PC3, and PC5 of PCA3.3 were redistributed across adjacent PCs of PCA4.1 (Table A4.2).

For 20 HWUE and 15 LWUE genotypes tested, a large similarity was observed in the behavior of most traits between Experiments 1 and 2 under imposed drought (Table A4.2). A paired t-test was also performed to compare cultivar means of SDW, WUE, OP, and, RDW_D of 35 selected genotypes between Experiment 1 and

2. It was found that the p-value of trait means between two groups was greater than the 95% significance threshold ($p > 0.05$) indicating that the key trait performance was consistent across seasons as presented in Figure 4.3 below. It should be noted that WUE was generally lower (or WU was higher) in the same germplasm in Experiment 2 than in Experiment 1 (Figure 4.3). However, similar cultivar-within-WUE group effects were seen for the major traits including WUE at $p < 0.0001$ in both experiments, with Trojan cultivar often separating from Samson and Nui cultivars (Figure 4.3).

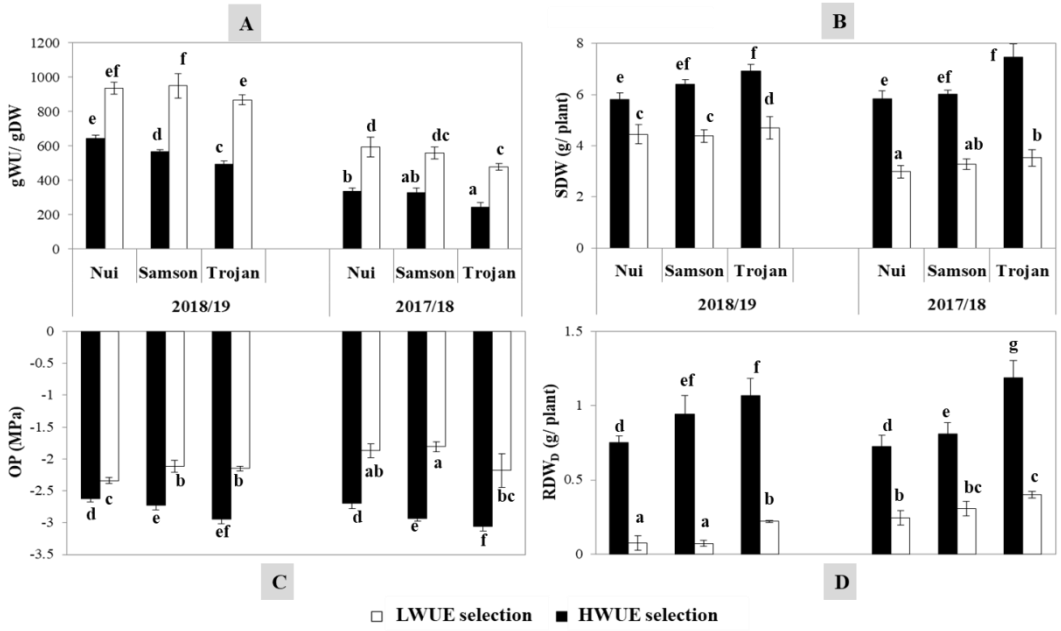

Figure 4.3 Relative changes in cultivar means of (A) water-use efficiency (g WU/g DM), (B) shoot dry weight (SDW), (C) leaf osmotic potential (OP), and (D) root dry weight at 20−50 cm depth (RDW_D) of Nui, Samson, and Trojan cultivars within high water-use (HWUE) and low water-use (LWUE) selections at M2 (moderate drought; 55−65% FC) in Experiment 2 (summer 2018/19) and Experiment 1 (summer 2017/18). Error bars are LSDs for cultivar trait means of HWUE and LWUE selections in each season; Bars with letters in common in each graph do not differ statistically ($p = 0.05$) and vice versa in the ANOVA.

4.4.2 Trait expression between and within HWUE and LWUE selections across the progressively developing soil moisture deficit imposed in Experiment 2

There were significant cultivar differences within both HWUE and LWUE selections for measured traits at each soil moisture stage imposed in Experiment 2 ($p < 0.05$). For example, WUE averaged for the HWUE and LWUE groups were

567±16 g WU/g DM and 918±44 g WU/g DM, respectively, at the key measurement stage (M2; 55–65% FC). There was a 61% increase in the mean WUE of the HWUE group compared to that of the LWUE group, with an increase of approximately 45%, 70%, and 80% in Nui, Samson, and Trojan cultivar means between groups, respectively, for WUE at both M2 and M3 (Figure 4.4A). A similar trait expression was seen for SDW of three test cultivars of HWUE and LWUE groups across M1, M2, and, M3 (Figure 4.4B). In particular, mean SDW of HWUE and LWUE groups were 6.4±0.23 and 4.5±0.36 g/plant, respectively at M2 (Figure 4.4B).

Leaf elongation and tiller appearance rates showed a gradual decrease as soil moisture declined throughout Experiment 2. However, the rate of decline was less in the HWUE group than in the LWUE group (Figure 4.5). Moreover, the cultivar Trojan amongst the three test cultivars showed the highest LER (Figure 4.5A) and TAR (Figure 4.5B) together with the least leaf senescence rate at each soil moisture stage. Cultivar means for RGS of the HWUE group were significantly higher than those of the LWUE group ($p < 0.05$), with the highest RGS among the three tested cultivars observed in the cultivar Trojan (Figure 4.6).

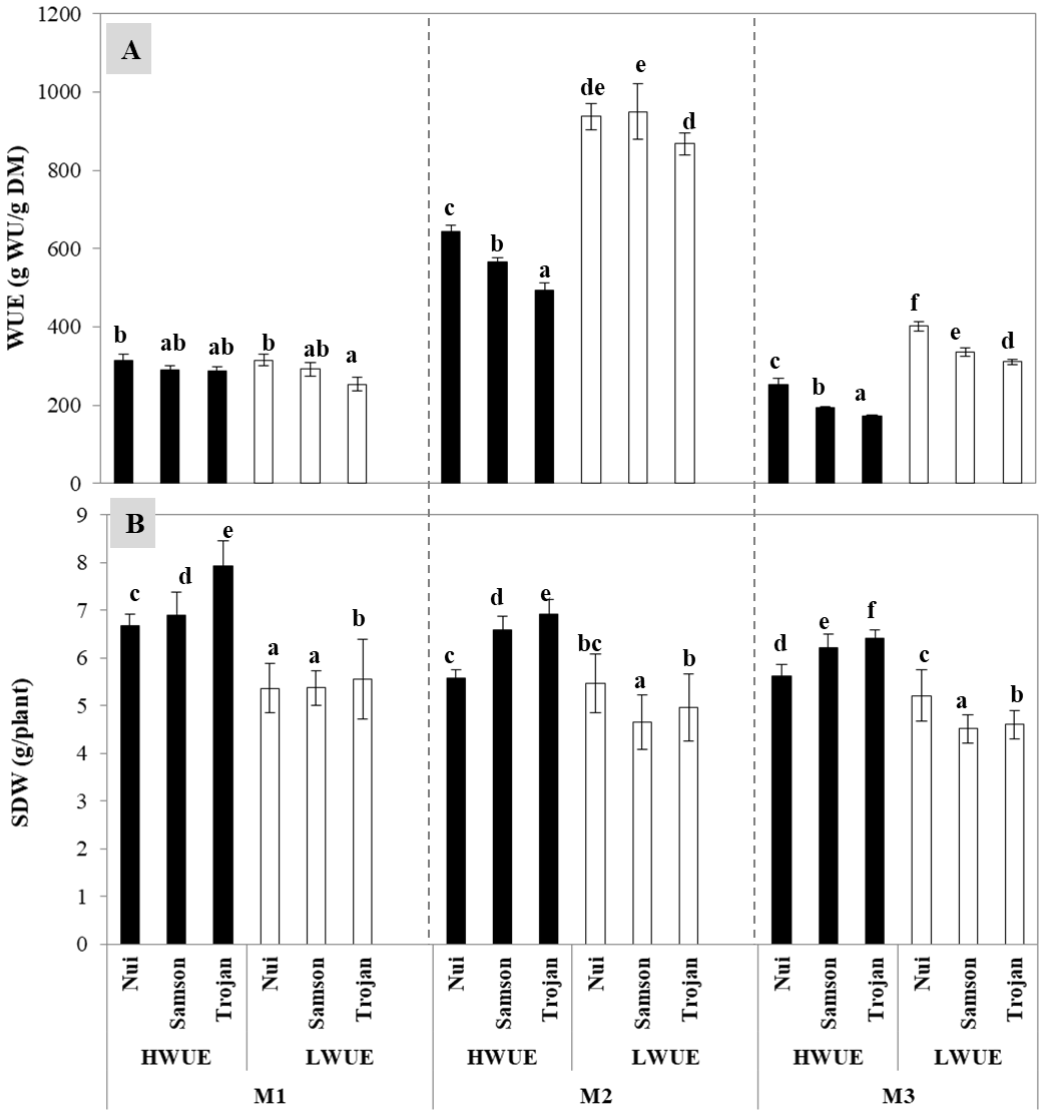

Figure 4.4 Change in (A) water-use efficiency (WUE; g WU/g DM) and (B) shoot dry weight (SDW) of three perennial ryegrass cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across three different soil moisture stages imposed in Experiment 2. (Note: measurements taken at 75–85%, 55–65%, and 45–55% of field capacity are denoted M1, M2, and M3, respectively). Error bars are LSDs for cultivar trait means of HWUE and LWUE selections at each measurement phase; bars with letters in common in each graph do not differ statistically (p = 0.05) and vice versa in the ANOVA.

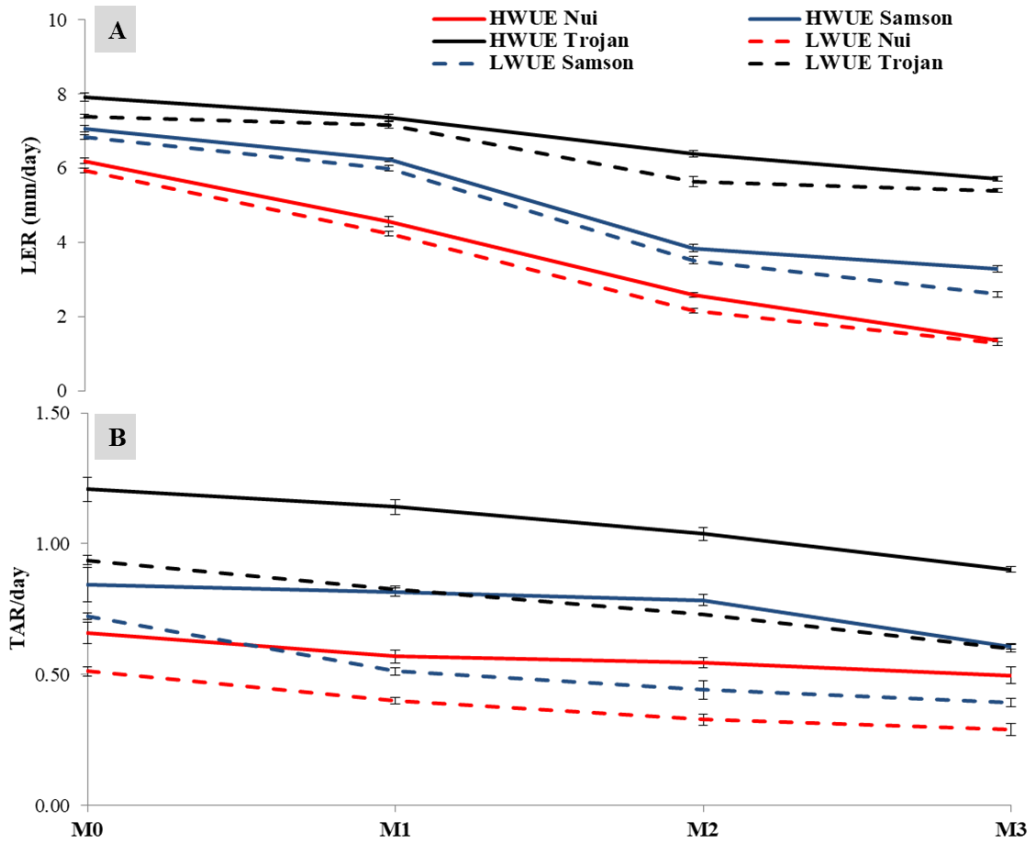

Figure 4.5 Change in (A) leaf elongation rate (LER) and (B) tiller appearance rate (TAR) of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across four different soil moisture stages imposed in Experiment 2 (Note: measurements taken at >95%, 75–85%, 55–65%, and 45–55% of field capacity are denoted M0, M1, M2, and M3, respectively). Error bars for each measurement phase are SEMs from ANOVA (Proc GLM) in randomized complete blocks and are shown where differences ($p < 0.05$) were detected among low and high water use (HWUE and LWUE, respectively) genotypes of Nui, Samson, and Trojan cultivars.

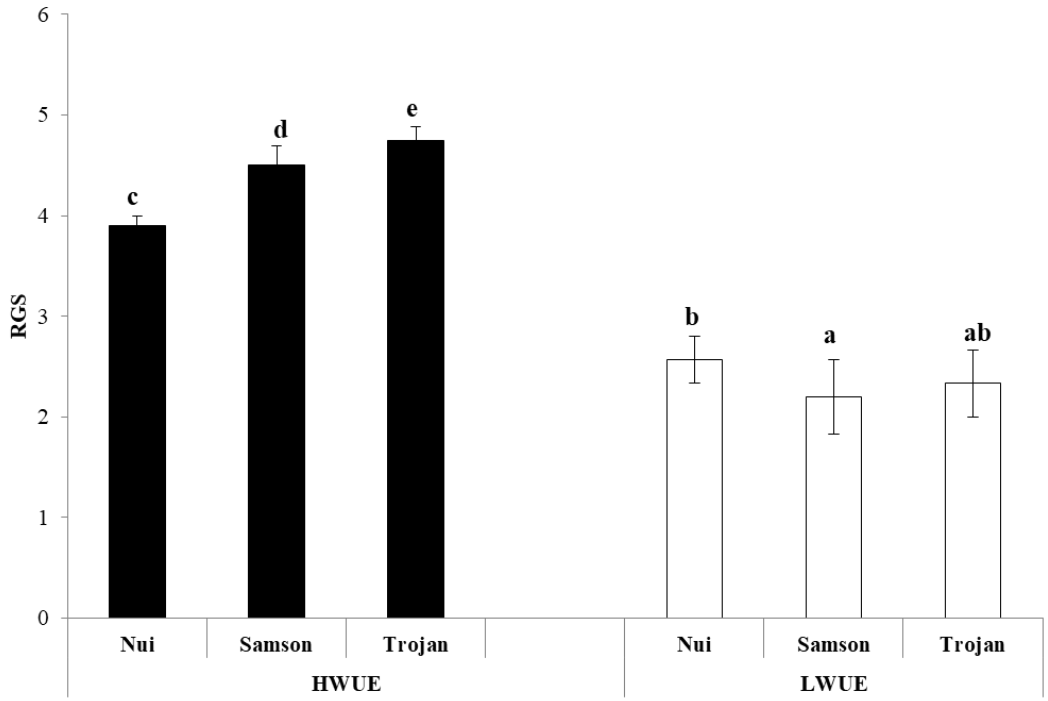

Figure 4.6 Post-cutting regrowth score (RGS; 0–5) of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) selections as recorded when watering was completely withdrawn at the end of M3 (45–55% field capacity) and genotypes were defoliated leaving 5 cm of stubble to support regrowth, drawing on residual soil moisture and scored after seven days in Experiment 2. Error bars are LSDs for cultivar trait means of HWUE and LWUE selections; Bars with letters in common do not differ statistically (p = 0.05) and vice versa in the ANOVA.

Cultivar means for RDW_T and RDW_D of the divergent HWUE and LWUE selections, together with means for SMC_T and SMC_D are shown in Figure 4.7. Notably, in M2, M3, and post-drought stages, HWUE genotypes exhibited greater deep rootedness and lower top root biomass compared to LWUE genotypes of each test cultivar. In general SMC is conserved in the HWUE selection and drawdown of SMC is not increased in proportion to higher SDW shown in Fig. 4.3B. Moreover, conservation of SMC was more pronounced in Trojan cultivar compared to that of Nui and Samson cultivars (Figure 4.7).

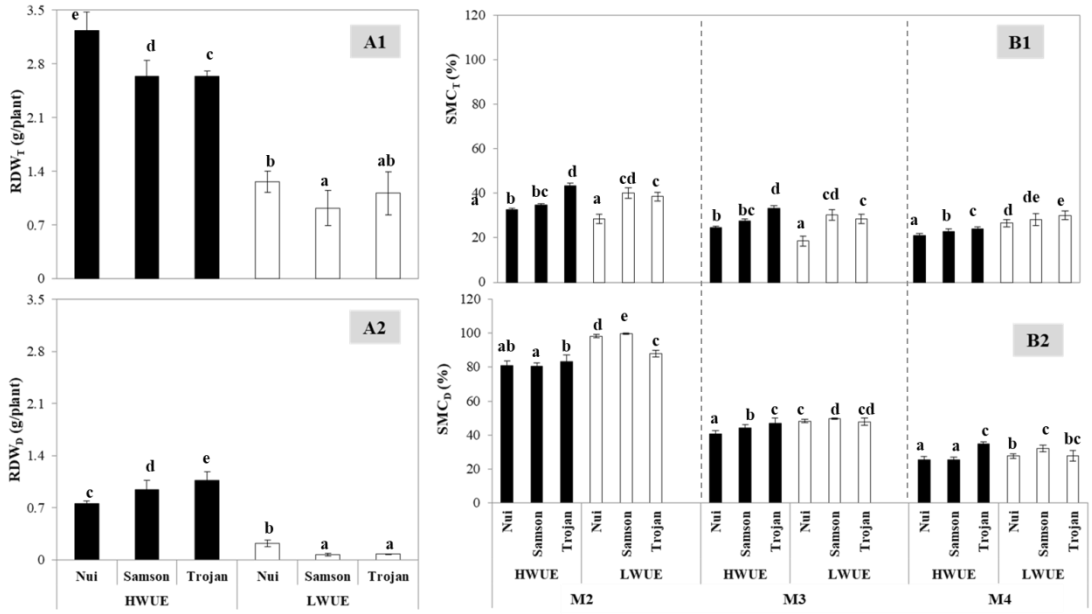

Figure 4.7 (A1) Root dry weight for 0–20 cm soil depth (RDW_T), **(A2)** Root dry weight for 20–50 cm soil depth (RDW_D) (Note: roots were harvested once at the end of the experiment) and **(B1)** gravimetric soil moisture content for 10–20 cm soil depth (SMC_T), **(B2)** gravimetric soil moisture content for 40–50 cm soil depth (SMC_D) across three progressively reducing (M2, M3, and M4) soil moisture levels (Note: measurements taken at 55–65% and 45–55% field capacity levels and post-cutting regrowth stage at the end of the drought treatments are denoted by M2, M3, and M4, respectively) as recorded for Nui, Samson, and Trojan cultivars of low water use (HWUE) and high water use (LWUE) selections in Experiment 2. Error bars are LSDs for cultivar trait means of HWUE and LWUE selections at each measurement phase; bars with letters in common in each graph do not differ statistically ($p = 0.05$) and vice versa in the ANOVA.

Low WU genotypes exhibited more negative OP (at low LWP) but similar RWC compared to LWUE genotypes across different soil moisture stages that was more pronounced at M2 than at M1 or M3 stages (Figure 4.8). The cultivar Trojan showed the greatest degree of OP reduction in all stages of the moisture deficit (Figure 4.8B).

As soil moisture declined in Experiment 2, the concentrations of WSCs and proline concentrations increased rapidly, with the highest HMW WSC levels and lowest proline levels observed in Trojan at M1, M2, and M3 (Figure 4.9A). In particular,

HWUE genotypes had higher HMW WSC concentrations than that LWUE genotypes and that trait response was less noticeable for LMW WSC and proline accumulated in leaf tissues (Figure 4.9B and 4.9C). Moreover, among the measured compounds of potential osmotic significance (i.e. LMWWSC, HMWWSC, and proline), HMW WSC had the strongest correlation with OP (details are presented in Section 4.4.2 below and Table 4.A3).

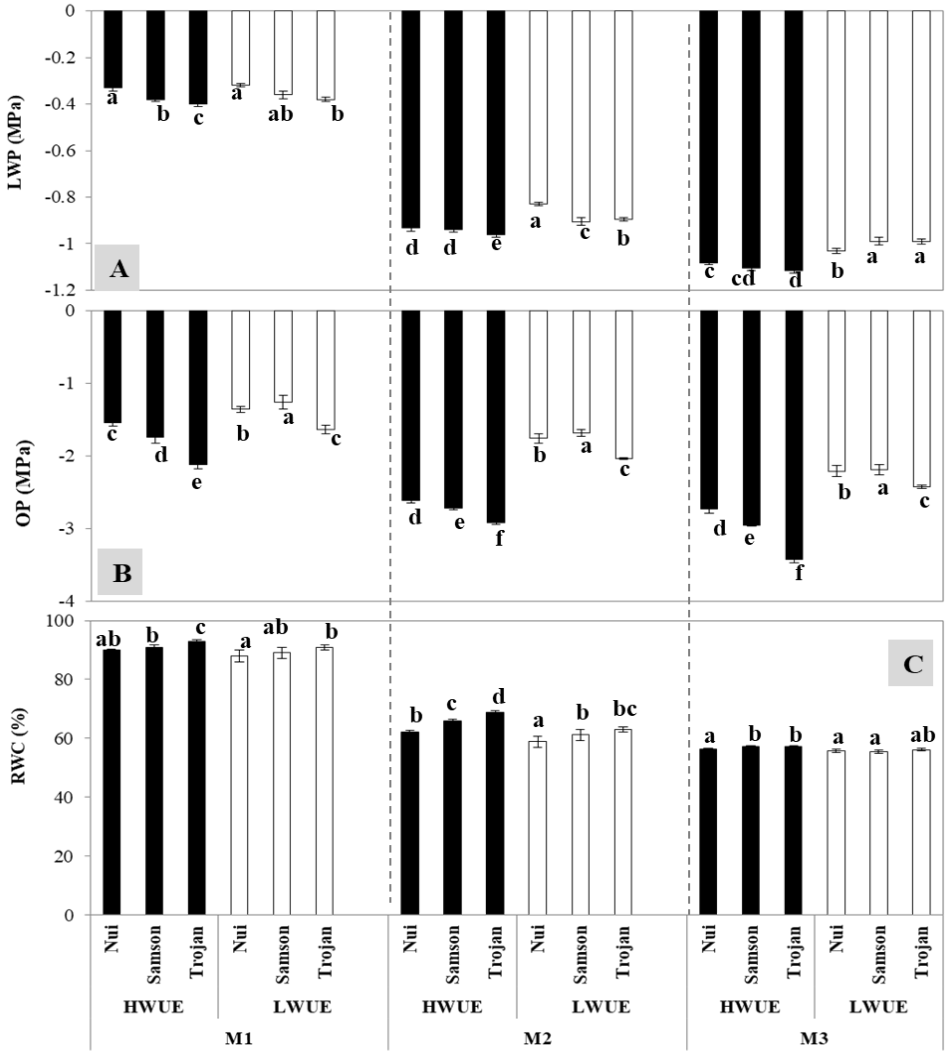

Figure 4.8 Change in (A) predawn leaf water potential (LWP), (B) leaf osmotic potential (OP), and (C) leaf relative water content (RWC) of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across the three different soil moisture deficit stages imposed in Experiment 2. (Note: measurements taken at 75–85%, 55–65%, and 45–55% field capacity levels are denoted M1, M2, and M3, respectively.) Error bars are LSDs for cultivar trait means of HWUE and LWUE selections at each measurement phase; bars with letters in common in each graph do not differ statistically (p = 0.05) and vice versa in the ANOVA.

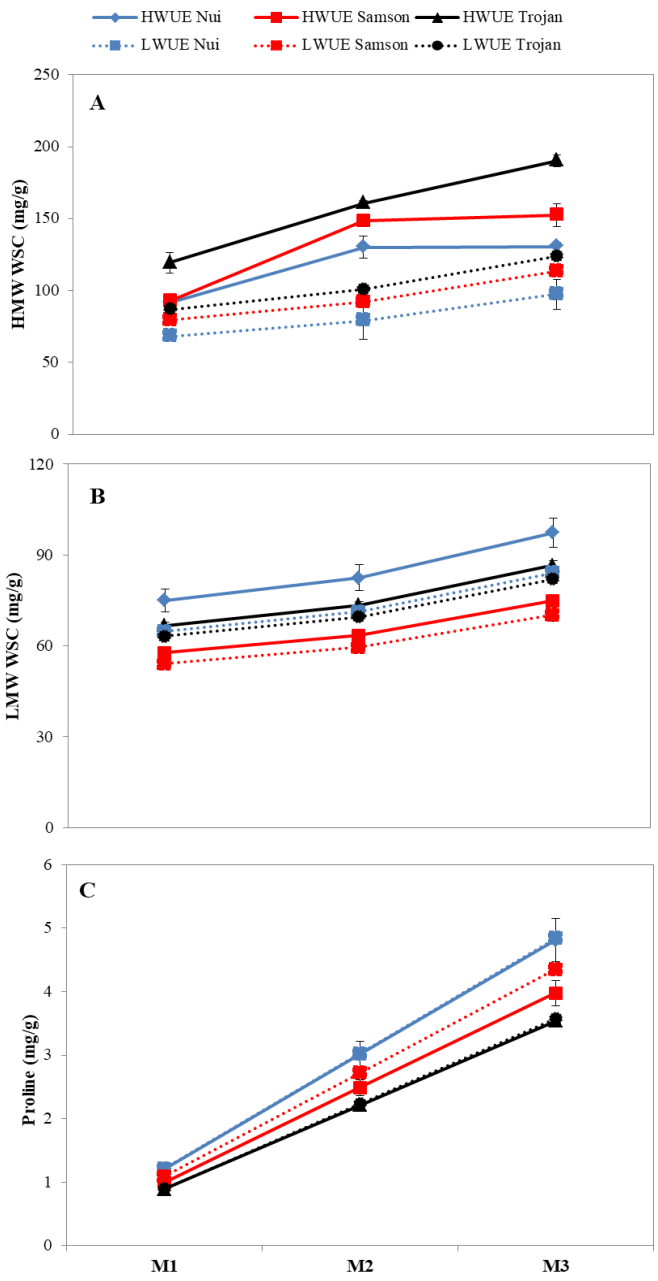

Figure 4.9 Patterns of change in concentrations of (A) high molecular weight water soluble carbohydrates (HMWWSC), (B) low molecular weight water soluble carbohydrates (LMWWSC), and (C) proline of leaf samples of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across three different soil moisture deficit stages imposed in Experiment 2. (Note: measurements taken at 75–85%, 55–65%, and 45–55% field capacity levels are denoted M1, M2, and M3, respectively.) Error bars for each measurement phase are SEMs from ANOVA (Proc GLM) in randomized complete blocks and are shown where differences ($p < 0.05$) were detected among HWUE and LWUE genotypes of three cultivars.

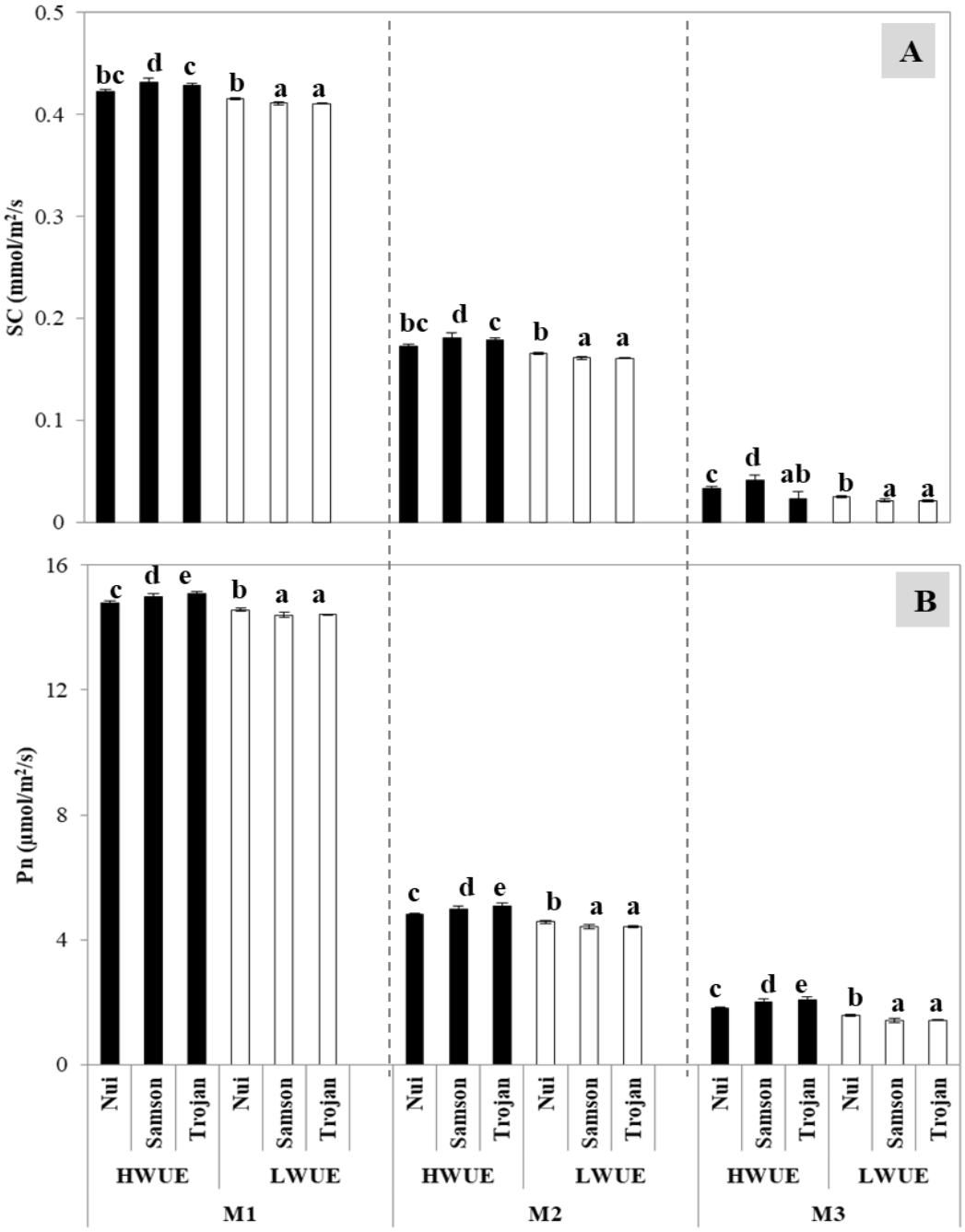

Figure 4.10 Change in (A) net photosynthesis (Pn) and (B) stomatal conductance (SC) of three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups across three different soil moisture deficit stages imposed in Experiment 2 (Note: 75–85%, 55–65%, and 45–55% field capacity levels are denoted M1, M2, and M3, respectively). Error bars are LSDs for cultivar trait means of HWUE and LWUE selections at each measurement phase; bars with letters in common in each graph do not differ statistically (p = 0.05) and vice versa in the ANOVA.

Photosynthetic rate and SC were also higher in HWUE genotypes than in LWUE genotypes of all three cultivars in all soil moisture stages (Figure 4.10). However, the cultivar effect for gas exchange measurements was less significant in both selections in Experiment 2 ($p > 0.05$).

Chlorophyll fluorescence (CF) parameters including Fv/Fm, F'v/F'm, φPSII, qP, NPQ, and ETR were measured once (at 35–45% FC; M3) in Experiment 2. The HWUE selection showed higher quantum efficiency and quantum yield of photosystem II and also higher qP (with lower NPQ) compared to that of the LWUE selection under intense drought conditions (Figure 4.11). Moreover, there were significant cultivar effects within groups for measured CF parameters similar to those of the key water relations traits measured in Experiment 2 ($p < 0.05$).

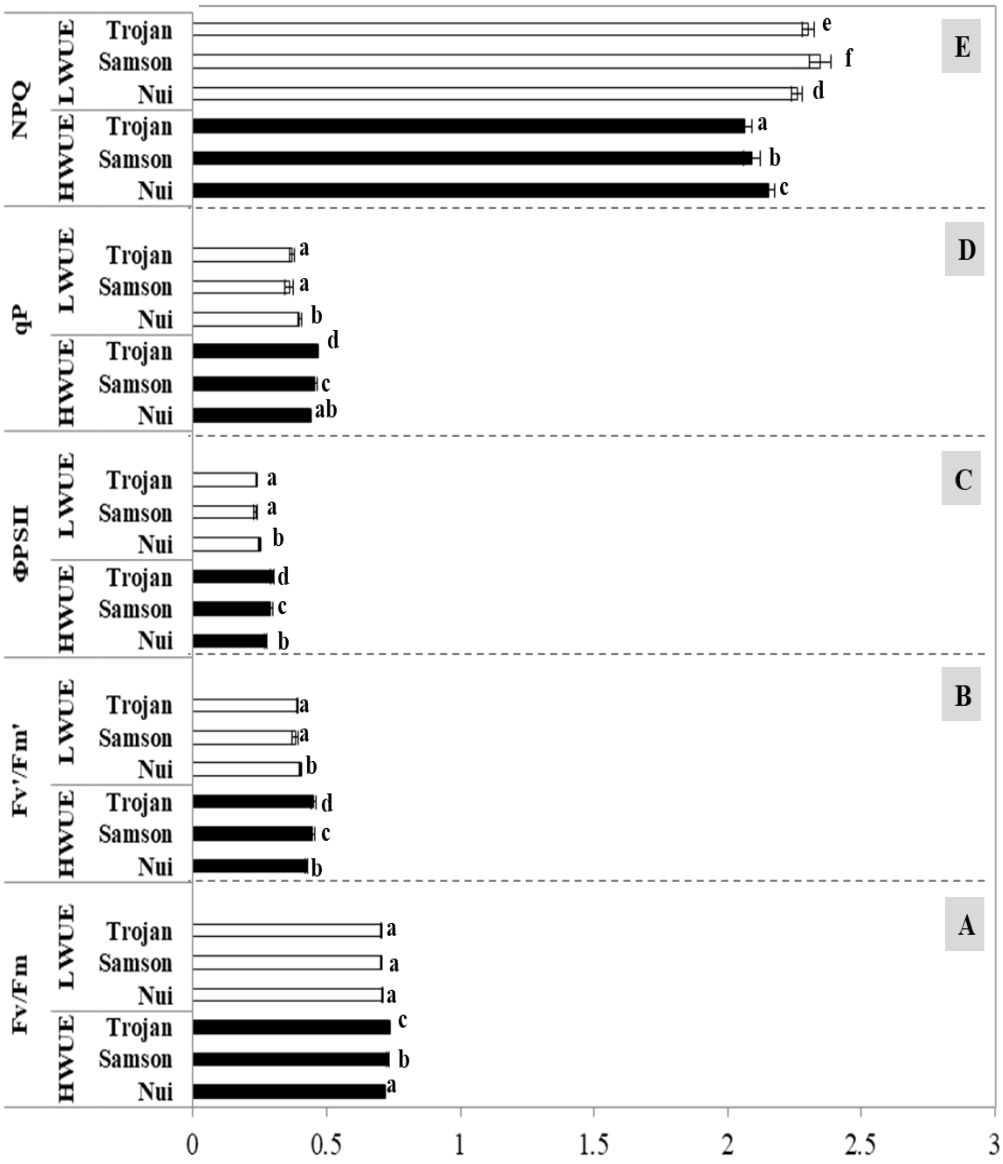

Figure 4.11 The key chlorophyll fluorescence measurements representing the photochemistry and the overall photosynthetic capacity of the three test cultivars (Nui, Samson, and Trojan) of low water use (HWUE) and high water use (LWUE) groups under intense drought (M3) in Experiment 2 (Trait abbreviations: (A) Fv/Fm, the maximum potential quantum efficiency of photosystem II; (B)F'v/F'm, the actual quantum efficiency of photosystem II; (C) φPSII, quantum yield of photosystem II; (D) qP, photochemical quenching; (E) NPQ, non-photochemical quenching). Error bars are LSDs for cultivar trait means of HWUE and LWUE selections; bars with letters in common in each graph do not differ statistically (p = 0.05) and vice versa in the ANOVA.

4.5 Discussion

4.5.1 Confirmation of trait associations identified in Experiment 1

An obvious measure of consistency of trait expression is the Pearson correlation between Experiments 1 and 2 for the individual trait data. For measurements conducted at M2 (or 55–65% FC), the trait correlations across experiments ranked in order OP > SDW > RDW_D > WUE > LWP > RGS > SMC_D > RWC > RDW_T ranging numerically from 0.910 ($p < 0.001$) to 0.302 ($p = 0.078$) with a strong agreement in six of the nine traits at $p < 0.01$ (Table 4.A4).

From an industry perspective the key point of interest was high WUE (or low g WU/g DM). An unexpected result in Experiment 1 was that two superficially very similar WUE-OP trait associations were identified in PC1 and PC3 of PCA3.1 (Table 3.2), PC1 linked to SMC_D depletion and PC3 to SMC_D conservation, respectively. The latter was considered of interest for further research and occurred in a subset of PRG genotypes. Encouragingly, from a plant breeding perspective, this complex SDW-OP-WUE trait association defined in PC3 of PCA 3.1 on which the HWUE divergent selection was made, and linked to increased RGS and RDW_D and conserved SMC_D, was extremely consistent in repeated measurement on the same genotypes in Experiment 2. This was objectively indicated by the high correlation of PC1 scores between PCA4.1 and PCA3.3 ($r = 0.880$, $p < 0.0001$, Table 4.A2), and by the closely similar patterns of means for key traits exhibited in Figure 4.3. It was salient to note that the comparatively low proportion of variation explained by PC3 of PCA 3.1 (i.e. 13.4%; Table 3.1) when 220 genotypes were considered, was elevated to 68.2% in PC1 of PCA4.1 (and to 60.9% in PCA 3.3), when only data for the selected 35 HWUE and LWUE genotypes were considered in compiling PCA4.1 (Table 4.1) and PCA 3.3 (Table 4.A1) in Experiment 2. This shows that the mentioned trait association is a major feature of the behaviour of the selected genotypes even though a minority of the 220 original PRG genotypes expressed this trait association. This is an important point that would be missed by many statisticians because it is generally considered that a lower-order PC (eigenvalue <1) indicating 13.4% of variation explained, as seen in PC3 of PCA 3.1 would be a fairly minor data feature that should not be pursued. In many cases, PCA results are presented as a biplot of PCs 1 and 2 with lower-order PCs not considered.

To the contrary, this trait association is identified as a major feature of the behaviour of the selected genotypes studied in Experiment 2. The confirmation of consistency across growing seasons from comparing PCAs 3.1 and 4.1 is also intuitively supported by visual inspection of the relative changes in cultivar means of WUE and OP data in both HWUE and LWUE selections across years (Figure 4.3).

As mentioned above, PC1 of PCA 3.1 that re-emerged as PC3 in PCA 4.1 involved traits suggesting increased extraction and use of soil water at depth by elite plants (as indicated by a negative coefficient for SMC_D) and was characterized (Chapter 3.4.2) as matching Blum's 'effective use of water' scenario where WUE is high because growth is not slowed down under imposed drought (Blum, 2009). PC3 of PCA 3.1 that re-emerged as PC1 of PCA 4.1, by contrast, is a 'true WUE' trait association because SMC_D is conserved at similar SDW, and appears to be a physiological scenario not recognized by Blum (2009) in his discussion of plant breeding methodologies for improving crop drought tolerance. Similarly, it is interesting to note that physiological models of PRG WU developed at Wageningen University such as LINGRA (Matthew et al., 2012) have no provision for varying WUE in the manner indicated by the SMC_D-conserving trait association, but instead regard WUE as a physiological constant. The fact that the PC1 and PC3 trait associations of PCA3.1 re-emerge weakly and strongly, respectively, in PCA4.1 was logical, and provides a further confirmation of the consistency of trait expression across Experiments 1 and 2. This is because divergent selection on PC3 of PCA3.1 in Experiment 1 was deliberately conducted to promote the trait association defined in that PC and to be the primary source of variation within the divergent selection in PCA4.1 and also to demote the trait association defined in PC1 of PCA3.1 in PCA4.1 as noted above. However, the HWUE and LWUE plants divergently selected on scores for PC3 of PCA3.1 should still exhibit variation in scores for PC1, which would emerge somewhere in PCA4.1 (i.e. PC3). From an agronomic perspective it is useful to note that these two different WUE-OP trait associations are independent of each other, since it is a mathematical property of PCA that scores for each PC have a zero correlation with scores of other PCs. The role of Pn, SC, CF parameters such as F_v/F_m, and WSCs in these two SDW-WUE-OP trait associations with contrasting SMC_D depletion and SMC_D conservation is discussed below.

Both Experiments 1 and 2 had in common a trait association a trait association that was explained by PC2 with a strong correlation of PC scores ($r = 0.781$, $p < 0.01$, Table 4.A2). This trait association was defined by a higher WUE in smaller plants without more negative OP (i.e. PC coefficients for WUE and OP have the same sign as SDW rather than opposite signs as seen in PCs 1 and 3) and also by a lower RWC coupled with less negative LWP, compared to PC1 and PC3, suggesting plants are experiencing physiological dehydration. This effect illustrates the reason for the concern raised by Blum (2009) that selection for high WUE may result in reduced yield. Again, the high WUE of small plants indicated in PC2 is independent of the two WUE-OP trait associations because different PCs in a PCA are independent of each other. It may be that improved WUE is an intrinsic side effect of comparatively small plant size, and if so this indicates a need to consider traits such as SDW and OP along with WUE if using WUE as a selection criterion, so as to select in the current experiment, PC3-type plants (or PC1-type plants if preferred) and reject PC2-type plants. It is reassuring, however, that this third trait association pattern for plants to differ in the strategy by which high WUE is achieved, is a stable feature of the data in both Experiments 1 and 2.

While OP is generally measured in leaves, roots also adjust osmotically (Blum, 2011; Velázquez-Márquez et al., 2015) that drives a WP gradient between the sink and the source and in turn generates a continuous supply of solutes or assimilates to the sink (i.e. solute transport from mature shoots to extending deeper roots) (Blum, 2017). In the current research, PC3-type plants specifically exhibited positive and negative PC coefficients for SDW and RDW_D and RDW_T, respectively, in both PCA4.2 and PCA4.1. It was speculated that a physiological trait response similar to aforementioned response may well have promoted preferential root growth for efficient root water capture from 0–20 cm and 20–50 cm soil layers in a restrained manner in the current research. It was found that cellular functioning of the lateral fibrous roots is generally affected while longitudinal growth of roots including the tap root continues to progress under severe water deficit, possibly because the zones of cell elongation and differentiation are located near the root apex and assimilates are naturally directed towards them under stress (Velázquez-Márquez et al., 2015). Thus, it can be suggested that the SMC_D-conserving WUE-OP-RDW_D trait association would drive moisture and solute gradients within the plant system that

also promotes assimilate partitioning for the growing tissues in the absence of gas exchange of elite plants. In previous research, WUE was found to be linked to 'root water uptake efficiency (or water consumption per root weight)' of a wheat crop grown under simulated drought stress conditions (Fan et al., 2008).

While the importance of turgor maintenance by OA for the drought recovery of plants growing in drying soils is largely affirmed in the literature, the relevance for net assimilation (Serraj & Sinclair, 2002) and yield gain is often questioned (Blum, 2011, 2017; Blum & Tuberosa, 2018). For PRG, one study found that more negative OP did not increase SDW and LER (Thomas, 1990) while a recent study suggested the importance of OA for yield gain (Cyriac et al., 2018). However, none of the earlier studies on PRG has explored a wider range of plant traits as defined by the SDW-WUE-OP-RDW_D/SMC_D trait associations under a progressively increasing soil moisture deficit extended for an adequate time period to facilitate substantial physiological adjustment, as seen in this study. In this experiment, under the conditions tested (Section 4.3), it was found that both types of SDW-WUE-OP-RDW_D/SMC_D trait associations may contribute to high shoot DM production through the maintenance of higher LER, TAR and, RGS either at low or high soil water consumption in elite plants.

4.5.2 Trait expression changes with water deficit intensification over time

While destructive measurements such as SDW could be performed only once within each regrowth cycle and RDW only once at the end of the experiment, non-destructive measurements (i.e. LER and TAR, LWP, OP and RWC, proline, leaf WSC levels, and gas exchange parameters) were measured as a time series with water deficit intensification and the results are informative as to the drought response pattern of the tested germplasm.

Previous research has discussed the role of OA (taken as represented by OP in this chapter) or the retention of leaf turgor in the soil moisture extraction by deeper roots in order to maintain assimilation (implying at most a minor reduction in stomatal conductance) and to support leaf appearance and leaf elongation at the expense of stored soil moisture in a water deficit (Blum, 2011; Morgan, 1984; Sharp & Davies, 1979; Wasson et al., 2012). Although the data in Experiment 2 are for plants of the SMC_D-conserving trait association in Experiment 1 selected as

HWUE plants, and not for plants with the SMC_D-depleting trait association that accounted for the major proportion of data variation in Experiment 1 (as indicated by PCA eigenvalues), such an effect was seen, where OP is progressively more negative both across the cultivar time series, and across measurements M1 to M3 in the present experiment (Figure 4.8). Linked with this, drought impact on LER from M0 to M3 was seen with a progression across the cultivar time series, being most pronounced in the oldest cultivar, Nui, and least pronounced in the most recently released cultivar, Trojan. Interestingly though, Nui maintained better TAR than Trojan across the water deficit intensification from M0 to M3 (Figure 4.5). Physiologically, increase in proline concentration with time as water deficit progressed was more pronounced in Nui than in Trojan and intermediate in Samson (Figure 4.9), indicating that more negative OP acts to alleviate physiological drought stress in these populations.

It seems that all mentioned drought-response patterns on exposure to increasing moisture deficit demonstrated by the SMC_D-conserving plant genotypes would also be shared by SMC_D-depleting plant genotypes. This may be due to the fact that both plant types share a similar pattern of coefficients for the SDW-WUE-OP-RDW_D component of the trait response, indicating a greater access to soil water in deeper soil layers through more negative OP and increased RDW below 20 cm soil depth, with some degree of resulting drought escape. It is helpful to understand that this is the drought response pattern operative in current New Zealand PRG cultivars with the ranking for expression of these traits Trojan > Samson > Nui suggesting an apparent breeding progression over the cultivar time series.

4.5.3 The role of gas exchange and CF in the SDW-WUE-OP trait association

The literature suggests that increase in stomatal resistance may account for more than 90% of the decrease in Pn in drought-exposed plants (Basu et al., 2016; Flexas et al., 2004; Tezara et al., 1999; Yan et al., 2017; Zait & Schwartz, 2018) as it is the earliest plant response to occur under drought (Faralli et al., 2019; Flexas et al., 2002; Quarrie & Jones, 1979). Similarly, the gradual soil moisture deficit imposed from M1 to M3 in the current experiment caused an overall decline in SC and Pn of both HWUE and LWUE genotypes of three cultivars (approximately 90%) (Figure

4.10). However, reduction in SDW at successive cuts from M1 to M3 was small in comparison, and was less than 20% (Figure 4.4).

As noted in Section 2.2.5.4, OA and gas exchange are closely associated plant physiological mechanisms under prolonged drought conditions. Theoretically, turgor maintenance by more negative OP can keep stomatal guard cells open for an undisturbed gas exchange and net assimilation (Farooq et al., 2009; Teulat et al., 1997) thus leading to yield gain under drought (Sanders & Arndt, 2012; Teulat et al., 1997). Equally, more negative OP can support partial stomatal closure for maintaining leaf turgor (McCree & Richardson, 1987; Medina & Gilbert, 2015). However, in this experiment, it was notable, when comparing PCs that defined the two different SDW-WUE-OP-RDW_D trait associations, that the SMC_D-depleting trait association (PC1 in PCA4.2 (Table 4.1) identified in Table 4.2 as the closest match to PC3 in PCA 4.1) had positive coefficients for Pn, SC, and CF parameters such as Fv/Fm (with decreased NPQ) and was now neutral for influence of SMC_D in the trait association. On the contrary, the SMC_D-conserving trait association (PC3 in PCA4.2 (Table 4.1) identified in Table 4.2 as the closest match to PC1 in PCA 4.1) had contrasting negative coefficients for Pn, SC, and CF parameters like Fv/Fm and (with a small positive PC coefficient for photochemical quenching) and a larger positive PC coefficient increased from 0.143 to 0.405 for SMC_D conservation. Thus, it appears that the inclusion of additional gas exchange and CF parameters in the PCA has provided an important clarification of the differences between the SDW-WUE-OP-RDW_D SMC_D-depleting and SMC_D-conserving trait associations. The association of the SMC_D-depleting trait association with increased Pn, SC, and Fv/Fm is consistent with earlier characterization of this trait with Blum's concept of effective use of water (Blum, 2009). Meanwhile, the finding that the SMC_D-conserving trait association displays many of the characteristics of 'effective use of water' such as high SDW and RDW_D and more negative OP, yet with a contrasting reduced Pn, SC, and Fv/Fm, raises questions as to the mechanisms at play in this trait association. An obvious candidate drought response pattern to explain this aspect of the and SMC_D-conserving SDW-WUE-OP-RDW_D trait association is reduced stomatal aperture, but if this were the explanation it would raise further questions of how plants with this trait association still maintain substrate-demanding traits like high SDW, RDW, WSC, and more negative OP. Clearly further research

on these questions is required even though some interesting possibilities are emerging in recent literature. For example, some research has shown that the role of OA is well-identified in plants grown under severe drought stress conditions (Blum, 2017; Sinclair & Ludlow, 1986) at which gas exchange is typically restricted depending on non-hydraulic or hormonal plant signals (Fan et al., 2008). Hence, it has been proposed that SC may be a valuable selection criterion for selecting plants for stress tolerance, if plants are selected under non-limiting moisture conditions (Faralli et al., 2019; Rebetzke et al., 2001). Meanwhile, in another development López et al. (2021) noted that pre-dawn transpiration governed by a plant's circadian rhythm can load leaf tissue with CO_2, resulting in high Pn in the early morning and enhanced drought tolerance. In addition, Flexas et al. (2016) asserted that the maintenance of high Pn with improved WUE under water stress may be a result of increased mesophyll conductance or biochemical efficiency of elite plants.

Apart from stomatal limitations, gas exchange physiology and plant growth may also be affected by non-stomatal limitations, particularly when plants are exposed to a long-term intensifying drought or repetitive drought cycles (Cielniak et al., 2006; Limousin et al., 2010; Yang et al., 2019), during which CF parameters may indicate the relative ability of a germplasm line to withstand the stress (Maxwell & Johnson, 2000; Zlatev & Yordanov, 2004). One question that has to be considered with respect to the SMC_D-depleting SDW-WUE-OP-RDW_D 'effective use of water' trait association is at what point low SMC in deeper soil layers mediated by this trait association that may lead to restriction Pn and growth processes and then to ineffective use of water, as a result. Clearly the SMC_D-conserving trait association should allow plants to continue active growth for some additional days under a developing drought, and also result in plants being less physiologically stressed. Therefore, careful attention is needed when considering WUE as a selection criterion for drought tolerance in PRG, to discern which of at least three potential trait associations (i.e. PCs 1–3 in PCA 4.2) is operative in a particular genotype, particularly when both production and survival are to be kept together as breeding objectives.

In general, Fv/Fm, F'v/F'm, φPSII, qP, and ETR of HWUE genotypes were generally higher while NPQ was lower than that of the LWUE selection (Figure 4.11). For example, HWUE genotypes of cultivar Trojan exhibited the highest

values for Fv/Fm, F'v/F'm, φPSII, qP, and ETR and the lowest value for NPQ, displaying its drought tolerance property irrespective of its ability to conserve soil moisture in a drought. A similar trait behaviour pattern was reported in earlier research where NPQ was negatively associated, and Fv/Fm, F'v/F'm, φPSII, and qP were positively associated with drought tolerance of *Lolium-Festuca* hybrids under severe drought (Cielniak et al., 2006). Also, Li et al. (2006) suggested that CF parameters are reliable indicators for screening a germplasm for drought tolerance in severe drought conditions and current results suggest that those could potentially be used to distinguish between SMC_D-depleting SMC_D-conserving genotypes for which the SDW-WUE-OP-RDW_D trait association has been established by other measurements.

As set out in Chapter 2, WUE can be measured as actual WUE or intrinsic WUE (WUEi), depending on the context, and equipment available. Intrinsic WUE, has been researched the most, and can be directly conferred by low SC and photosynthetic capacity of plants (Fischer et al., 1998; Nakhforoosh et al., 2015; Passioura, 2006). However, it is a well-known fact that upscaling from WUEi to whole plant or actual WUE is highly complex (Hsiao et al., 2007; Laffray & Louguet, 1990). For example, genotypic differences observed in SC of soybean were closely associated with those of WUEi but were uncorrelated with WUE under both drought and well-watered situations (Gilbert et al., 2011). However, such information related to PRG selection experiments is almost absent. There is a gap in the pasture breeding research in that the efficacy of WUEi as a proxy for direct measurement of WUE has received little evaluation, perhaps because it has been confirmed to be reasonably representative of gas exchange measurements when defining drought tolerance of many studied plants (Ebdon & Kopp, 2004; Ghannoum et al., 2002). However, this component was a gap in this research that needs attention in future research.

4.5.4 The role of osmotically-active substances in the SDW-WUE-OP trait association

Volaire et al. (1998) found that traits that were strongly associated with superior survival of cocksfoot and Mediterranean PRG species under prolonged summer drought are (a) deep rootedness for greater water uptake, (b) more negative OP (at

more negative LWP) of growing leaf tissues, (c) large pool-size of fructans such as HMWWSC with degree of polymerization >4, and (d) low accumulation of proline in stubble. In the current results, it was found that HWUE plants of cultivar Trojan displayed similar trait behaviour particularly, (a), (b), and (c) above, but also lower shoot proline concentrations than Nui or Samson, under imposed drought in this experiment (Figures 4.4–4.9). Presumably, lower proline levels denote lower physiological stress.

As noted in Chapter 2, previous studies mostly viewed the role of WSCs as osmotically-active substances in plant cells that are important for the post-drought survival and plant persistence under severe drought conditions. The literature also suggests that assimilate partitioning between plant parts is generally signaled by WSC accumulation under drought (Blum, 2011; Koch, 1996; Smeekens, 1998). Blum (2017) asserted that photosynthetic products, mainly simple sugars, are accumulated in leaf cells first and then, transferred to below-ground plant parts to induce OA in root cells (Chimenti et al., 2006; Morgan, 1984; Roland et al., 2015). As discussed above, deep rooting is stimulated as a result of OA in root cells in a moisture deficit. Also, Sandrin et al. (2006) found that the metabolism of elongating leaf blades was triggered by the accumulation of HMW sugars (mostly fructans) in two tested cool-season grass species. PCA coefficients (Table 4.1) show that both SMC_D-depleting and SMC_D-conserving SDW-WUE-OP-RDW_D trait associations have modest positive coefficients for LER and TAR, and with HMWWSC concentration (PC-coefficients +0.200 and +0.261, respectively, of PCA4.2; Table 4.1) though not with LMWWSC or proline. Thus, the data indicate that HMWWSC accumulation is associated with both the RDW_D-conserving and RDW_D-depleting SDW-WUE-OP trait associations. Also, WUE, SDW, LER, TAR, RGS, RDW_D, and OP traits were significantly correlated with HMW WSC of elite genotypes measured at M2 or 55–65% FC ($r > 0.5$, $p < 0.05$; Table 4.A3). In contrast, correlations between LMW WSC and most other traits were not significant ($p > 0.05$) except for the positive correlation observed between LMW WSC and OP ($r = 0.246$, $p = 0.050$; Table 4.A3). Also, it was found that HWUE genotypes of cultivar Trojan had higher levels of HMWWSC than Nui or Samson at M1, M2 and M3, whereas LMW WSC was the highest in Nui (Figure 4.9). Linking the high HMW WSC levels in Trojan in Experiment 2 to drought response is logically reasonable because the HWUE

genotypes of ‘Trojan’ consistently displayed more pronounced drought tolerance properties in Experiment 1 than genotypes of Samson or Nui. A possible physiological role for the enhanced accumulation of HMW WSC in the more drought tolerant genotypes might be the use of the HMW WSC as an energy reserve to degrade to LMW WSC as osmolytes as required to lower OP as drought progressed.

In support of the apparently major contributing role of HMW WSC accumulation under imposed drought to PRG drought tolerance in this experiment, Amiard et al. (2003) reported that drought tolerance of two-month old potted PRG accessions was strongly associated with the accumulation of long-chain fructans (degree of polymerization 3–8) in leaf tissues, in a two-week simulated drought. Furthermore, in two field trials, accumulation of HMW WSC (mostly HMW fructans) was found to contribute to pasture recovery under summer drought conditions (Thomas & James, 1999; Turner et al., 2008). Even so, several authors have discussed the complexity of metabolic pathways by which different WSC molecules are formed and partitioned within and between plant cells, and have suggested that plant selection for WSC under stress may generate unforeseen deleterious physiological effects (Humphreys, 1989b; 1989c; Rogers et al., 2019; Shahidi et al., 2017; Turner et al., 2008). Research to identify desirable carbohydrate concentration profiles deserves greater attention in forage grass breeding programs because plant WSC has long been identified as a heritable trait (Humphreys, 1989a) that also contributes to the nutritive value (Edwards et al., 2007) in drought-tolerant PRG genotypes. From another perspective, sugar accumulation in plants is known to facilitate assimilate transportation from source (i.e. mature roots and leaves) to sink organs (i.e. extending roots or expanding leaves) and such transfer that in turn upregulates genes responsible for both the continuation of Pn and further remobilization of assimilates to growing tissues (Koch, 1996; Smeekens, 1998). However, none of the previous studies reported evidence for the importance of WSC accumulation for gas exchange except in the case of post-drought recovery. Similarly, for the elite genotypes of the current experiment, it was found that the traits displaying the highest correlations with HMW WSC ($r > 0.7$) were, in descending order: OP ($r = 0.927$), WUE, RGS, LWP, Pn, and SC (Table 4.A3). Taking everything into account, it is clear that both

the SMC_D-depleting and SMC_D-conserving SDW-WUE-OP-RDW_D trait associations are supported physiologically by HMW WSC accumulation.

4.6 Conclusions

Experiment 2 retested 15 high-WU (LWUE) and 20 low-WU (HWUE) PRG genotypes, divergently selected in Experiment 1 from the Nui-Samson-Trojan cultivar time series, for consistency of trait expression under imposed soil drought in a second growing season. Shoot WSC and proline concentrations, and CF parameters were additionally evaluated in Experiment 2. Results showed that the key trait data correlated well between Experiments 1 and 2. In particular, SMC_D-depleting and SMC_D-conserving plant types distinguished among others with the SDW-WUE-OP-RDW_D trait association by PCA in Experiment 1 were reconfirmed in Experiment 2. The consistent results on retesting engendered confidence that the key drought tolerance traits identified in Experiment 1 could be used as selection criteria in a PRG breeding program, subject to confirmation of heritability.

A PCA including the additional traits not measured in Experiment 1 established that the SMC_D-conserving SDW-WUE-OP-RDW_D trait association was closely and functionally linked to the accumulation of HMW WSC (but not with accumulation of LMWWSC or proline) in the shoots of elite genotypes. This PCA also showed that the SMC_D-depleting SDW-WUE-OP-RDW_D trait association had positive coefficients for Pn, SC, and CF parameters like Fv/Fm, whereas the SMC_D-conserving trait association displayed contrasting negative coefficients for those traits. This confirms the characterisation of those trait associations as ‘effective use of water’ as outlined by Blum (2009) and true WUE, respectively. More research is required but a possible mechanism to explain the SMC_D-conserving SDW-WUE-OP-RDW_D trait association is transpiration reduction through partial stomatal closure. Independently of these two trait associations, another PC showed that small plants may also display high WUE. Hence, direct selection for WUE without considering the associated traits such as OP, RDW, SMC, and SDW would be inadvisable.

Chapter 5

Investigation of drought response patterns in two PRG commercial breeding lines of contrasting origin and comparison with the HWUE selection from Experiments 1 and 2

5.1 Abstract

This experiment (Experiment 3) investigated WUE trait associations in an advanced commercial breeding line (CBL, 38 genotypes) and a seed line derived from crossing Mediterranean and Middle Eastern seed accessions (MMEL, 14 genotypes). As before, two clonal ramets of CBL and MMEL genotypes were grown in 55 cm tall pots in a glasshouse environment and pots of Experiment 3 were inter-randomised with those of HWUE genotypes in Experiment 2. After three months of root development near field capacity (FC), test genotypes were exposed to a four-week period of progressively increasing moisture deficit and at 60% FC, SDW, RDW_T, RDW_D, RGS, WUE, LWP, OP, RWC, SMC_T, SMC_D, Pn, and SC trait measurements were performed for analysis. Pot WU of individual genotypes ranged from 160 to 990 g WU per g DM across CBL and MMEL lines, demonstrating a range that would support selecting each germplasm for WUE. To explore key drought response patterns associated with WUE, PCA 5.1 and PCA 5.2, respectively, were prepared for the averaged trait data of the CBL and MMEL. PC1 of PCAs 5.1 and 5.2 explained 52.6% and 61.6% of data variation, respectively, with generally similar trait associations in the two populations but also with some key differences. Common features included a trait association between increased WUE, SDW, RDW_D, RWC, and more negative OP. CBL genotypes with this trait association had low RDW_T, high RGS, and conserved SMC_D similar to the key trait association demonstrated by the HWUE selection made from the Nui-Samson-Trojan population in previous experiments. In contrast, MMEL genotypes had high RDW_T, low RGS and depleted SMC_D. When a PCA was performed on stacked data considering the three populations as a single population (PCA5.3), the trait association represented by PC1 largely followed that of the CBL in PCA5.1 and ANOVA of PC scores showed that PC1 scores did not differ significantly between CBL, HWUE, and MMEL germplasm lines, whereas scores for PC2 and PC3

strongly discriminated between the three germplasm lines for SDW, RDW_T, LWP, and RWC traits. Lastly, a PCA (PCA 5.4) was compiled using the nine key trait measurements of the 38 CBL genotypes from the glasshouse experiment together with the seed company's overall field performance scores from national and Manawatu trials, making a total of 11 trait attributes. Notably, PC1 explaining 43.1% of data variation was closely similar to PC1 of PCA5.3 and had near zero association between field scores and glasshouse water relations data, while PC2 explaining 18.3% of data variation, strongly captured information from company field scores and indicated a modest association between the field scores and SDW, RDW_T, LWP, and RWC traits, as judged by PC coefficients in the first two PCs of PCA 5.4. Thus, these results suggest that the field evaluation system could benefit from consideration of externally-measured water relations traits, including WUE, as selection traits for PRG drought tolerance.

5.2 Introduction

The success of improving forage species for drought tolerance has always been limited due to plant breeders' incomplete knowledge on different water deficit tolerance strategies in different geographic ecotypes and also limited opportunities for them to work closely with plant ecophysiology or stress physiology research (Johnson & Asay, 1993). As noted in Chapter 4, precise and repeatable selection techniques should be developed and verified through quality scientific research. Outcomes of such research could be an incentive for breeders to reorient their selection focus and reallocate resources efficiently in existing commercial breeding programs, if the relative gain of the new selection traits is higher than easy-to-measure agronomic traits (Lee et al., 2012). This also implies the importance of keeping research collaborations between plant scientists and breeders in pasture improvement programs. In order to achieve this, knowledge on key physiological trait responses that are associated with pasture drought tolerance should be transferred into realistic breeding objectives. Thus, this chapter provides details including methodology, results and discussion and, conclusions of Experiment 3, that was conducted in collaboration with a commercial seed company in New Zealand and in parallel with Experiment 2 in summer 2018/19, to explore ways to bridge the research gap mentioned above.

The current experiment tested the practical usefulness of the methodology used in Experiments 1 and 2 by including elite PRG genotypes from two contrasting company-commercial breeding lines together with the HWUE and LWUE selections from previous experiments in a common Experiment. Given that the first aim of this experiment was to compare water relations trait associations of the two company-commercial lines with each other and with the HWUE/LWUE selection under imposed drought. The performance of the HWUE and LWUE selections was separately reported in Chapter 4 (Experiment 2) and the consistency with Experiment 1 results demonstrated. In this chapter, the data for the two company-commercial lines (Experiment 3), inter-randomised with plants in Experiment 2 are presented. The two specific germplasm lines that were used in this study were the elite genotypes (clonally replicated) from the company's cultivar development programme (commercial breeding line, CBL) and a company breeding line developed following inter-crossing between heat tolerant Mediterranean and Middle

Eastern PRG seed accessions and back-crossed to New Zealand material (Mediterranean-Middle Eastern cross, MMEL). However, the summer dormancy status of the incorporated MMEL was unclear. The second aim of this experiment was to compare the trait performance of the CBL-elite plants in Experiment 3 with the company's field scores for spaced plant performance of the same genotypes.

The results and discussion section comprises two main sections; Section 1 compares and contrasts the key drought-response patterns of different PRG populations tested and Section 2 discusses the alignment between glasshouse-measured water relations traits and commercial breeding field performance scores. At the end of the results and discussion section, the writer attempts to answer the question: "To what extent do results from this glasshouse testing align with those from field testing of the same set of genotypes?", so as to inform real-world applications in pasture breeding programs targeting improved drought tolerance, persistence, and summer yield of PRG.

5.3 Materials and methods

5.3.1 Plant material

Similar-sized clonal ramets (4–5 tillers each) of 38 CBL and 14 MMEL genotypes carrying the AR37 endophyte were obtained from the cultivar development programme of a New Zealand seed company as plant materials in Experiment 3.

5.3.2 Experimental set up & drought treatments

The experimental set up and drought treatments were similar to those of Experiment 2 (Sections 4.3.2 & 4.3.3). However, the measurements for CBL and MMEL plants were performed only once (i.e. at M2 or 55–65% FC) and these were compared with corresponding results for the HWUE selection at M2 in Experiment 2.

5.3.3 Measurements & analyses

Leaf water relation measurements (i.e. LWP, RWC, and OP) shoot and root dry weights (i.e. SDW, RDW_T, and RDW_D), RSR, regrowth score, pot WU, WUE, SMC_T, SMC_D, and gas exchange measurements (Pn and SC) were recorded at 55–65% FC as described in Chapter 4.3.3 above.

Averaged data from two replicates of the 38 and 14 genotypes, respectively, of the CBL and MMEL were used to generate two different PCAs (i.e. PCA5.1 and PCA5.2) in Minitab version 14 to establish drought-response patterns of each germplasm line. Next, stacked trait data for the 38 CBL, 20 HWUE, and 14 MMEL genotypes were used to compile a new PCA (PCA5.3) to compare and contrast key drought-response patterns of the CBL and MMEL germplasm lines against those of the HWUE selection. A fourth PCA (PCA 5.4) was employed to test the level of agreement between the trait performance in the current glasshouse experiment and field scores of the CBL obtained by the commercial company in their internal performance testing that was used to select genotypes for use in cultivar development.

Trait expression differences between the three tested germplasm lines were visualised in 'radar charts' (Figure 5.1), using the means for genotypes of each germplasm line of traits measured as described above at M2. To remove differences of scale between traits, trait data were standardised using the MS Excel "=Standardize" function across the three germplasm lines to be compared.

5.4 Results

5.4.1 Drought-response patterns of the three tested PRG germplasm lines

Examination of the trait associations within the CBL and MMEL was followed by a comparison across the three germplasm lines. Figure 5.1, using standardised data, provide a visual evaluation of ecophysiology differences observed in CBL and MMEL compared to the HWUE selection in Experiment 2. The untransformed trait means appear in Table 5.A1. The tested germplasm lines differed statistically for traits SDW, LWP, and RGS ($p < 0.001$ in each case); separation of other trait means was not statistically significant ($p > 0.05$).

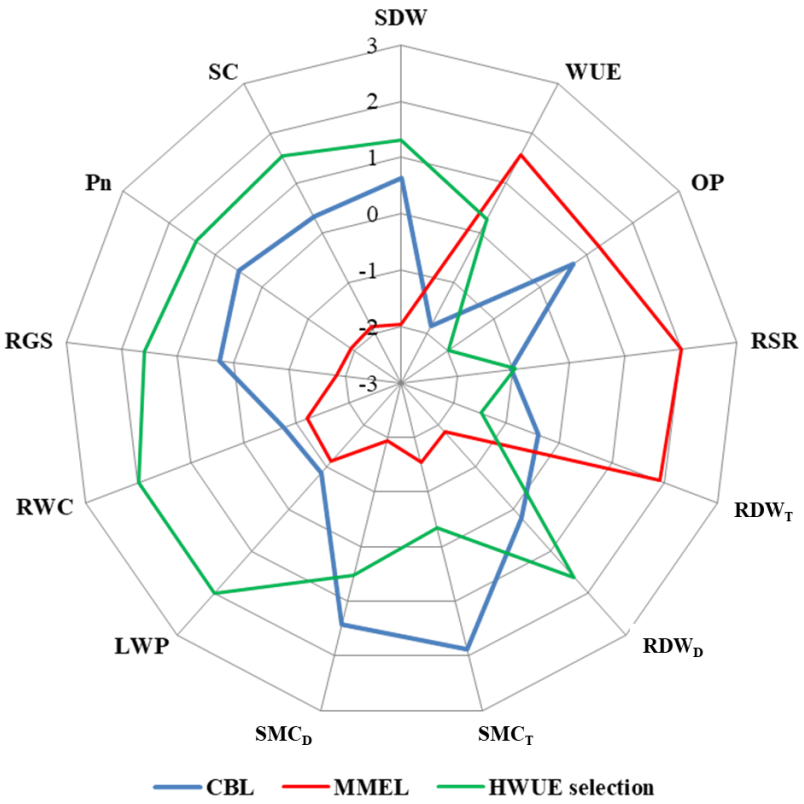

Figure 5.1 Radar chart comparing the trait behavior of commercial breeding line (CBL), Mediterranean cross (MMEL), and low water use or high water-use efficiency (HWUE) selection from Experiment 2 for shoot dry weight (SDW), water-use efficiency (WUE), leaf osmotic potential (OP), total root: total shoot ratio (RSR), root biomass at 0–20 cm depth (RDW_T), root biomass at 20–50 cm depth (RDW_D), gravimetric soil moisture content at 40–50 cm depth (SMC_D), predawn leaf water potential (LWP), leaf relative water content (RWC), post-cutting regrowth score (RGS), photosynthesis (Pn), and stomatal conductance (SC) under imposed drought (Note: Averaged trait means of two replicates of 38, 20, and 14 genotypes from CBL, MMEL, and HWUE populations, respectively, were standardized using the "=Standardize" function in MS Excel). Germplasm line differences for traits SDW, LWP and RGS are statistically significant at $p<0.001$.

For PCA 5.1 and PCA 5.2, the first three PCs explained, respectively, 79.7% and 92.5%, of the total variation and had eigenvalues > 1 (Jolliffe, 2003), and were selected from the nine PCs available from PCA of nine measured water relations traits in the CBL and MMEL. The first PC of both PCA5.1 and PCA5.2 explained more than half of the total variance (52.6% and 61.6%, respectively). In PCA5.1

(CBL), PC1 indicated a very similar pattern of trait associations to that in PC1 of PCA3.3 and PCA4.1 (also PC3 of PCAs 3.1 and 4.2; Chapter 3 and 4, respectively) with substantive positive coefficients for SDW, RDW_D, SMC_D, RWC, and to a lesser extent RGS, and substantive negative coefficients for WUE (i.e. high WUE), RDW_T and OP. Notably, there was also a positive PC1 coefficient for SMC_D in both PCAs. Trait associations in PC1 of PCA5.2 (MMEL) differed from PC1 of PCA5.1 (CBL), only in having a negative coefficient for RGS. PC2 of both PCAs had large coefficients (>0.600) for RGS and substantive positive coefficients for SDW but contrasting coefficients for RDW_T (positive in MMEL and negative in CBL). PC3 of both PCAs featured coefficients near +0.800 for LWP, positively associated with RWC and RDW_T (Table 5.1).

Table 5.1 Eigenvalues and principal component (PC) coefficients for the first three PCs generated by PCA of nine morphological and water relations traits of 38 genotypes of the commercial breeding line (CBL, PCA 5.1) and 14 plants of the Mediterranean cross (MMEL, PCA 5.2) at M2 (55–65% FC). Coefficients less than 0.100 are suppressed.

	PCA 5.1 (CBL)			PCA 5.2 (MMEL)		
	PC1	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**
Eigenvalue	4.73	1.34	1.09	5.54	1.71	1.07
% Variation explained	52.6	15.1	12.1	61.6	19.1	11.8
% Cumulative variance	52.6	67.7	79.7	61.6	80.6	92.5
SDW	0.246	0.352	–	0.308	0.321	−0.317
WUE	−0.432	0.156	–	−0.417	−0.100	–
LWP	–	0.179	0.852	−0.164	−0.243	0.797
OP	−0.441	–	–	−0.415	–	−0.146
RWC	0.378	−0.181	0.314	0.343	–	0.333
SMC_D	0.422	−0.123	–	0.407	–	0.107
RDW_D	0.438	−0.125	–	0.409	–	0.190
RDW_T	−0.125	−0.457	0.369	−0.200	0.640	0.201
RGS	0.119	0.631	0.168	−0.203	−0.639	0.206

Trait abbreviations: SDW, Shoot dry weight; WUE, Water–use efficiency (g WU/g SDW); LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC, gravimetric soil moisture content at 30–40 cm depth; RDW_T, Root dry weight at 4–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth; RGS, Post–cutting regrowth score. (Note: Negative coefficients for WUE indicate less WU/g SDW).

In PCA5.3 compiled from the data depicted in Figure 5.1 (i.e. data for the nine measured morphological and water relations traits of CBL, HWUE, and MMEL genotypes) in stacked columns and the first five PCs (explaining 93.8% of data variance) of the nine available PCs are presented (Table 5.2). Relationships identified by PCs 1–5 explained 48.3%, 22.8% and 10.8%, 7.9%, and 3.9%, respectively, of data variation. Stacking the data for the three germplasm lines in PCA 5.3 resulted in a PC1 with trait associations largely following those of the CBL in PCA5.1, including a positive coefficient indicating conservation of SMC_D, but with the main difference that the influence of traits SDW, LWP, and RGS was diminished (as indicated by coefficients closer to zero). These three traits had a high influence (large coefficients) in a new SDW-LWP-RGS trait association in PC2. ANOVA of PC scores showed that PC1 scores did not differ significantly between CBL, HWUE, and MMEL germplasm lines ($p > 0.05$), whereas scores for PC2 strongly discriminated between the three germplasm lines ($p < 0.0001$) (Table 5.2). Table 5.2 also shows that PC3, PC4, and PC5 had large coefficients for RDW_T, LWP, and RWC, respectively. ANOVA of PC scores also showed that the plant-to-plant variation in RDW_T identified in PC3 is present in all three tested germplasm lines (i.e. mean scores do not differ significantly between germplasm lines), whereas low LWP (i.e. positive coefficient and negative mean score, PC4) and high RWC (i.e. negative coefficient and negative mean score for PC5) are traits that significantly distinguish the CBL from the other two tested germplasm lines (Table 5.2).

Table 5.2 Eigenvalues and principal component (PC) coefficients and p-values in the ANOVA output on PC scores (including ANOVA mean scores for each PC of trait data from each germplasm tested) of the first five of nine PCs in PCA 5.3, compiled using stacked trait data of nine morphological and water relations traits in 38 commercial breeding line (CBL) genotypes, 20 low water use selection (HWUE) genotypes from Experiment 1, and 14 Mediterranean cross (MMEL) genotypes as measured at M2.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Eigenvalue	4.35	2.05	0.97	0.70	0.36
% variation explained	48.3	22.8	10.8	7.9	3.9
Cumulative % variance	48.3	71.2	82.0	89.9	93.8
WUE	–0.456	0.092	0.007	0.033	0.084
SDW	0.155	0.564	0.058	–0.413	–0.224
RDW_T	–0.102	–0.193	0.938	–0.064	0.201
RDW_D	0.452	–0.016	–0.076	–0.091	0.317
SMC_D	0.448	–0.062	– 0.006	–0.024	0.357
OP	–0.449	–0.030	–0.076	–0.058	–0.194
LWP	–0.036	0.471	0.133	0.854	–0.024
RWC	0.381	–0.186	0.220	0.116	–0.795
RGS	–0.072	0.612	0.382	–0.264	–0.070
CBL mean score	0.119	–0.079	–0.031	–0.414	–0.223
MMEL mean score	–0.557	–2.092	–0.039	0.602	0.234
HWUE mean score	0.163	1.614	0.086	0.365	0.259
p-value	ns	< 0.001	ns	< 0.001	< 0.01

Trait abbreviations: SDW, Shoot dry weight; WUE, Water–use efficiency (g WU/g SDW); LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC, gravimetric soil moisture content at 30–40 cm depth; RDW_T, Root dry weight at 4–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth; RGS, Post–cutting regrowth score (Note: Negative coefficients for WUE indicate less WU per gram of SDW).

5.4.2 Investigating alignment between glasshouse-measured water relations traits and commercial breeding field performance scores.

PCA 5.4 (Table 5.3) included company field performance scores for the 38 CBL genotypes from their breeding programme, in addition to the traits measured in the glasshouse. Two field scores were included; the mean for trial sites all around New Zealand (MFS) and the score obtained from testing at Palmerston North (MPNS). In PCA 5.4, PC1 explained 43.1% of data variation, the pattern of coefficients for the nine water relations straits correlated closely with those of PC1 in PCA 5.3 ($r = 0.995$, $P < 0.001$) and the field scores had no influence with PC coefficients of PC1 for MFS and MPNS (i.e. –0.005 and –0.040, respectively). This result indicated that there is a strongly similar relationship between the PC1 of PCA 5.3 and PCA 5.4 irrespective of field performance scores. Meanwhile, PC2 of PCA 5.4 explained 18.3% of data variation, was strongly influenced by the field scores with coefficients of +0.654 for MFS and +0.579 for MPNS and showed some consonance with glasshouse data, especially for the traits SDW, RWC, $RDW_{T,}$ and LWP (i.e. PC-coefficients +0.200, +0.210, –0.232 and –0.296, respectively) (Table 5.3). For the lower-order PCs, PC3 in PCA 5.4 (12.2% variation explained) had substantive coefficients for traits RGS, SDW and LWP following PC2 in PCA5.3. PC4 in PCA5.4 (8.5% variation explained) had a substantive coefficient of +0.837 for LWP (resembling PC4 in PCA 5.3) but also displayed a large positive coefficient (+0.456) for MPNS. Lastly PC5 of PCA5.4 (7.6% variation explained) had substantive scores of +0.853 for RDW_T and +0.400 for RGS, resembling PC3 of PCA 5.3.

Table 5.3 Principal component (PC) eigenvalues and coefficients for the first five PCs of PCA5.4 that includes nine yield and water relations traits and two commercial company field test scores (MFS and MPNS, see below) of 38 perennial ryegrass genotypes of the commercial breeding line (CBL) compiled for the investigation of consonance between glasshouse and field testing. The first five PCs of 11 available, explaining 89.6% of data variation are presented.

	PC1	PC2	PC3	PC4	PC5
Eigen value	4.74	2.01	1.35	0.93	0.83
%Variation explained	43.1	18.3	12.2	8.5	7.6
%Cumulative variance	43.1	61.4	73.6	82.1	89.6
WUE	−0.433	0.067	0.141	0.040	0.054
SDW	0.245	0.200	0.480	0.061	0.193
RDW_T	−0.123	−0.232	−0.373	0.110	0.853
RDW_D	0.437	0.011	−0.135	0.039	−0.080
SMC_D	0.437	0.032	−0.136	0.020	−0.051
OP	−0.440	−0.066	0.038	−0.027	−0.126
LWP	−0.035	−0.296	0.315	0.837	−0.104
RWC	0.379	0.210	−0.110	0.199	0.092
RGS	0.120	−0.008	0.665	−0.158	0.400
MFS	−0.005	0.654	−0.052	0.087	0.147
MPNS	−0.040	0.579	−0.128	0.456	0.078

Trait abbreviations: SDW, Shoot dry weight; WUE, Water–use efficiency (g WU/g SDW); LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC, gravimetric soil moisture content at 30–40 cm depth; RDW_T, Root dry weight at 4–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth; RGS, Post–cutting regrowth score; MFS, Country-wide mean field score; MPNS, Palmerston North mean field score (Note: Negative coefficients for WUE indicate less WU/g SDW).

5.5 Discussion

5.5.1 The three tested germplasm lines all exhibited a trait association between more negative OP and high WUE, with conserved SMC_D under water deficit challenge

In this experiment, key trait performance of the HWUE selection, which in Experiments 1 and 2 had demonstrated high WUE, more negative OP, high SDW and regrowth, and higher proportion of RDW_D under similar drought conditions to the current experiment, was used as the reference for studying drought tolerance trait associations of CBL and MMEL. However, it should be noted that the HWUE genotypes are themselves an elite selection, so the experiment is a comparison of three elite lines, without a 'typical' or average benchmark. It was then important to impose a moisture deficit challenge event that was of sufficient intensity and duration to stimulate PRG physiological and morphological adjustments to a measurable level, for exploring drought-response patterns of three different germplasm lines tested. Based on experience with the earlier series of experiments, a drying down period of four weeks to reach 55–65% FC at M2 (and to reach a LWP of approximately –1.0 MPa) was imposed to meet this requirement in the current experiment.

As the key output in this experiment, it was found that results of PCAs 5.1 and 5.2 (i.e. compiled for CBL and MMEL, respectively) and also PCA5.3 (i.e. prepared using stacked data from all three germplasm lines) extracted a common drought tolerance trait signature or a pattern that represented the largest proportion of the total variation (i.e. PC1) to each germplasm line. More importantly, it was similar to the key trait response pattern that was identified by PC1 of PCA3.3 and PCA4.1 (also PC3 of PCA3.1 and PCA4.2) in previous experiments. Therefore, PC1 may be used to identify elite genotypes within each germplasm line that can generate high SDW coupled with higher soil moisture retention and regrowth and lower RDW_T at lower LWP levels while higher WUE, SDW, deep rootedness, and leaf hydration (i.e. high RWC and more negative OP at low LWP) and that this trait association is a shared feature in elite genotypes across the three tested germplasm lines. In Experiment 1 and 2, it was suggested that this drought tolerance trait response of the HWUE selection would make a viable selection criterion for screening a PRG

germplasm for the improvement of both the production and survival of PRG under imposed drought, if the selected traits are heritable in addition to the consistent trait expression observed by those genotypes across two growing seasons (See Table 4.A2). It was the same drought response pattern that was shown by PCA to identify top-performing genotypes of CBL (i.e. germplasm selected for 'summer performance', among other commercial selection criteria, through several generations) and MMEL (i.e. PRG genotypes from naturally arid regions) in terms of drought tolerance features. This implies that commercial PRG breeding programs that targeted pasture persistence may have indirectly achieved pyramiding of genes from WUE and associated traits in elite plants (i.e. more negative OP and increased RDW_D).

Previous research on so-called 'Mediterranean' PRG germplasm from dry environments has typically revealed a drought resistance strategy featuring summer dormancy (i.e. reduced SDW as a survival strategy under prolonged drought) to varying degrees. This seemingly reduced plant water demand is usually coupled with a deep rootedness that would increase moisture supply capability (Laude, 1953; Matthew et al., 2012; Nie & Norton, 2009; Norton et al., 2006). As noted above, the alignment of PC1 coefficients for MMEL in this experiment with those of HWUE and CBL plant selections differed from results obtained in previous studies of Mediterranean PRG varieties like Medea (developed in Adelaide; (Silsbury, 1961)), in which slower soil moisture drawdown together with deep rootedness were not featured. This similarity of 'between-genotype-within-germplasm line' trait associations of the three tested germplasm lines identified by PC1 of PCA 4.1 or PCA 3.3 for HWUE, PCA 5.1 for CBL, and PCA 5.2 for MMEL may have arisen from the common growing environment they shared when inter-randomized with Experiment 2 HWUE plants, although there is no obvious reason why this would happen. Another possibility is that introgression of a common drought-tolerant germplasm during the development by the commercial company of both the CBL and MMEL germplasm lines tested has occurred. Also, it is not known if the Middle Eastern germplasm that formed the starting point for the development of the MMEL has the same or different drought tolerance trait associations and mechanisms from the North African germplasm used in the development of Medea. Therefore, it was not known precisely how the MMEL line would respond to water deficit prior to

analyzing the current results. However inspection of germplasm line means (Table 5.A1) indicated that despite the common trait associations with the CBL and HWUE selection in PCA, the MMEL plants had a mean SDW less than 40% that of CBL plants and a comparatively poor RGS. Thus, it appears a measure of summer dormancy is present in the MMEL, despite introgression with non-dormant germplasm. While this summer dormancy may confer superior survival in a severe drought (Anderson et al., 1999; Easton et al., 2011), it is clear that the MMEL selection may not align with major breeding objectives of a New Zealand pasture breeder (i.e. breeding new cultivars for higher herbage yield and pasture persistence) (Lee et al., 2012; Matthew et al., 2012). This preference of New Zealand farmers for PRG varieties with capacity to retain growth in summer drought was also discussed in previous studies. For example, trials of the cultivar Medea developed from North African germplasm in Australia showed that the herbage productivity loss associated with summer dormant plant behaviour would be a major disincentive for New Zealand farmers (Hussain, 2013; Vartha, 1975).

It is interesting that all three tested PRG germplasm lines showed variability in RDW_T, accounting for 10.8% of total data variation and moderately associated with RGS. (PC3, Table 5.2). A plant with high RDW_T would be well placed to capture nutrients in shallow soil layers at times of the year when moisture supply is plentiful, such as nitrogen mineralization events after autumn rain. A similar phenomenon was explained in some literature where soil resource availability may be a better predictor of the root uptake efficiency of upper and deeper roots as those forage independently for different resources (Kulmatiski et al., 2017). It is also possible that top roots might provide reserves to support ongoing deeper root growth in moisture deficit. Morgan & Condon (1986) found that increased root mass or high RSR is a common drought adaptive response that is exhibited by drought-exposed plants in the absence of OA. Also, the highest proportion of the total root biomass and the greatest contribution to RSR of pasture species is contributed by roots growing at 0–15 cm depth (White & Snow, 2012). This raises further questions of: (1) how different drought tolerance strategies impact on plant performance through the rest of an annual summer-winter seasonal cycle and (2) whether different genotypes, that make up the population of genotypes found in a synthetic cultivar, have different specialist functions to optimize performance of the plant community

with shared resources at different times of the year. One of such strategies includes the relationship between defoliation or grazing and return of excreta by grazing animals to pasture fields. Further research would be needed to explore these questions.

In the PCA results, it was also noted that three tested germplasm lines were supposedly elite so there was no distinct benchmark for $RDW_{D.}$ Deeper root biomass was fairly high for all three germplasm lines (with a slightly higher value for the HWUE germplasm than the other two, which may arise from selection for a trait association that included high RDW_D) compared to that of the LWUE selection (i.e. average RDW_D values of LWUE, HWUE, CBL, and MMEL were 0.14±0.03, 0.95±0.07, 0.84±0.10, and 0.81±0.15, respectively), which was inter-randomized in the same glasshouse space (Figure 5.A1). From the current results and most published literature, it is clear that RDW_D and OP are functionally correlated drought tolerance traits. Therefore, it was expected that elite genotypes, that exhibited turgor maintenance by more negative OP, would also exhibit deep rootedness under imposed drought. Although it was apparent that the OP-RDW_D trait association contributed to efficient leaf WU in the current results, the question remains whether all three tested germplasm lines extend roots into deeper soil layers as a result of OA or due to some other mechanism. For example, deep rootedness has generally been identified as one of the major characteristics of summer dormancy in PRG germplasm lines that have a Mediterranean origin (Hussain, 2013; Lee et al., 2012; Silsbury, 1961). As noted above, MMEL used in this experiment that was not selected for summer dormancy by the commercial company that developed it still showed dormancy-related characteristics (i.e. low SDW). However, OP of all three germplasm lines tested was more negative than that of the LWUE selection (i.e. average OP values of LWUE, HWUE, CBL, and MMEL lines were –1.79±0.03, –2.77±0.03, –2.63±0.06, and –2.67±0.08, respectively). Similarly, Volaire et al. (1998) found traits that were strongly associated with superior survival of Mediterranean PRG species under prolonged summer drought conditions include both deep rootedness and negative OP of growing leaf tissues. This implies that the OP-RDW_D trait association in drought tolerant plants may not be distinguishable from that occurring in summer dormancy, if SDW and WUE traits are not simultaneously considered when selecting PRG for drought tolerance.

5.5.2 The extent of common information capture between glasshouse data and field scores

The need to get pasture breeders and scientists together to define morpho-physiological traits that positively influence pasture performance and productivity and can feasibly be used as selection criteria in breeding programs has long been recognised (Lee et al., 2012). Water-use efficiency (or its surrogate traits including OP) is an obvious candidate-trait for examination of the alignment between beneficial traits identified in ecophysiology research and the selection practices operated by commercial breeders. Thus, one aim in this Chapter was to compare the drought tolerance traits of different germplasm lines (Section 5.5.1 above). The second was to examine for the CBL germplasm line that had already been subjected to selection through several generations for various traits required for commercial success (Section 5.5.1). As an additional step, it was also planned to seek answers to the question: "Can the field performance evaluations performed by the commercial breeder be enhanced with information on WUE performance of PRG?", as measured by the methodology developed in this thesis.

Improving WUE has recently gained attention of crop researchers when breeding plant species for drought tolerance (de Almeida Silva et al., 2012; Feldman et al., 2018). As noted in Section 5.5.1 above, PC1 of PCA 5.3 defined a trait association found in all three PRG germplasm lines tested and involving efficient WU. Moreover, the results of Experiments 1 and 2 also established the importance of WUE as a selection trait for PRG drought tolerance through OP-WUE-SMC_D trait association in plants of the HWUE selection, and these were consistently expressed under similar drought conditions across two growing seasons (Chapter 4). Hence the trait association defined by PC1 shows a promise from an ecophysiology perspective of providing an avenue for improved plant performance on farm in summer. Even so, when field scores (i.e. MFS and MPNS) were added to the glasshouse data as additional plant traits and the PCA re-run, PC1 of the new PCA (PCA 5.4) was almost identical in structure of coefficients to PC1 of PCA 5.3, with the field scores having near zero influence. Instead, the information from field scores was largely captured in PC2, which by definition is uncorrelated with PC1. However, there was a link between field performance scores and glasshouse data in PC2, with high field scores reflected in improved glasshouse SDW and RWC at more negative LWP (i.e.

increased plant hydration) and also lower RDW_T (i.e. lower RSR as a sign of balanced DM partitioning) (PC2 in PCA 5.4, Table 5.3). These alignments in the data are intuitively logical, and considering the massive difference in growing environments, this level of consonance between field and glasshouse scores could be considered encouraging rather than discouraging (Limpens et al., 2012). There are many cases in the literature where consistency of agronomic data between experiments or between environments is similar to that seen here or even weaker (Forero et al., 2019; Khaembah et al., 2013; Limpens et al., 2012). Also, the field evaluation system is basically designed to capture traits such as yield potential, insect pest and disease resistance, and the presence of a strong root system to support PRG persistence in multi-year field trials. In addition, endophyte infection, absence of awns, and uniformity of heading date are also considered in the field scoring system. By contrast, the current experiment focused on beneficial morpho-physiological traits that may promote efficient WU and PRG drought tolerance.

The practical question that follows from consideration of the consonance between glasshouse and field scores above is that of selection from among the 38 CBL plants of those plants to be used as the foundation for a new cultivar. From this perspective, the field evaluation system did not capture information on PRG WU well. A plot of MPS against WUE made to explore this point showed no correlation ($r = 0.16$; $P > 0.05$; Figure 5.2). Specifically, the top-performing four genotypes in the Manawatu field trial, genotypes with MPNS > 6.0 (P17, P19, P21 and P37) had a wide range of WUE values. However, the rejection of P21 and P37 and selection instead of P9 and P32 would greatly improve the average trait performance of WUE and associated traits of the selection with little reduction in mean field performance score (Figure 5.3, below).

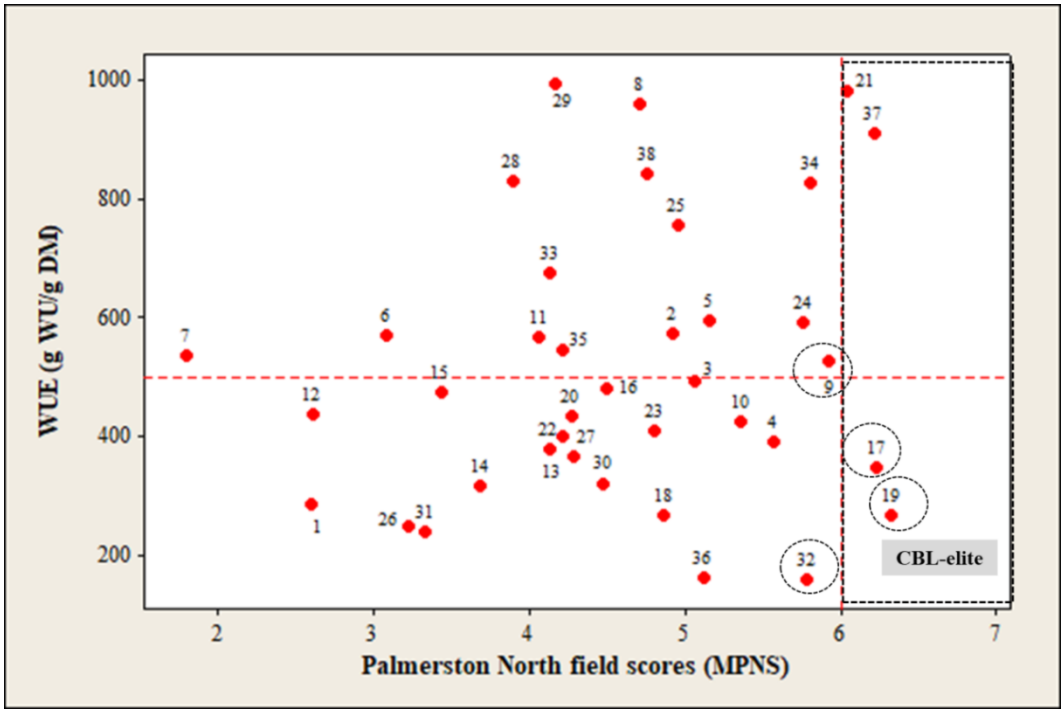

Figure 5.2 The distribution of water-use efficiency (WUE; g WU/g DM where higher values indicate low WUE and vice versa) of thirty eight genotypes of the commercial breeding line (CBL) in Experiment 3 against their Palmerston North field scores (MPNS). Red dotted lines identify genotypes with less than 500 g WU/g DM and field score higher than 6.0. Numbers in black are genotype identifiers. Regression line is not shown in the graph (y = 34.418x + 358.94; r=0.16, p>0.05) (Note: 'CBL-elite' plants, namely P32, P17, P19, and P10 indicate the commercial company has selected within the CBL based on a two-criterion selection of the best four CBL plants considering field score and glasshouse WUE; the figure also visually demonstrates how the rejection of P21 and P37 and selection instead of P9 and P32 would greatly improve mean WUE of the selection with little reduction in mean field performance score).

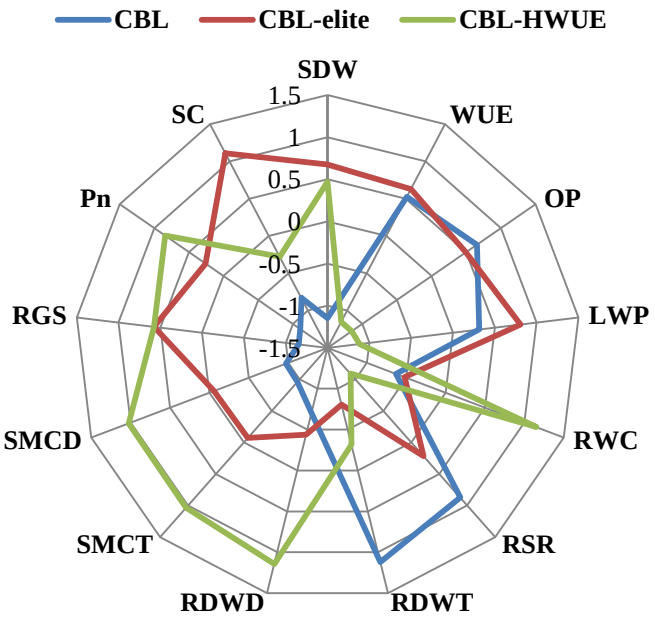

Figure 5.3 Radar chart illustrating the shift in the average trait performance in a two-criterion selection of the best four CBL plants considering Palmerston North field score (MPNS; as the prioritized selection criterion) and glasshouse WUE (CBL-elite) namely, P19, P17, P21, and P37 and the best four CBL plants considering high glasshouse WUE together with the highest possible MPNS (CBL-HWUE) namely, P19, P17, P32, and P9 for shoot dry weight (SDW), water-use efficiency (WUE), leaf osmotic potential (OP), root: shoot ratio (RSR), root biomass at 0–20 cm depth (RDW_T), root biomass at 20–50 cm depth (RDW_D), gravimetric soil moisture content at 40–50 cm depth (SMC_D), predawn leaf water potential (LWP), leaf relative water content (RWC), post-cutting regrowth score (RGS), photosynthesis (Pn), and stomatal conductance (SC), when they were selected from 38 genotypes of the commercial breeding line (CBL) under imposed drought in the glasshouse environment. Trait means of CBL (population mean), CBL-elite, and CBL-HWUE were significantly different at $p < 0.05$ for most traits presented in this figure except for LWP. Standardized values of the averaged trait means of two replicates of 38 genotypes of the CBL and 4 genotypes in each CBL-elite and CBL-HWUE are presented in this figure. The untransformed trait means for each mentioned group appear in Table 5.A1.

5.5 Conclusions

This experiment reports data from two clonal replicates of 38 and 14 PRG genotypes of an advanced commercial breeding line (that was improved with the introgression of a North African germplasm in the local germplasm for persistence; CBL) and a crossbred Mediterranean and Middle Eastern germplasm (that was selected from different Mediterranean ecotypes grown in naturally dry regions; MMEL), respectively, that were inter-randomised with the HWUE genotypes when retesting for consistency of trait expression across years, in Experiment 2. As analysed by PCA, the major trait associations (i.e. WUE-OP-RDW_D-RDW_T-SMC_D-RGS) among the yield and water relations data collected in this experiment were very similar across the three germplasm lines compared, namely the HWUE selection comprising elite plants of Nui, Samson and Trojan cultivars, and the CBL and MMEL elite plants from a commercial PRG breeding programme. The writer ascribes the similarity of drought response between CBL and MMEL lines to common germplasm introgressed with the founding germplasm in the breeding process. However, it is interesting to note that the CBL has SMC_D-conserving properties, in contrast to the majority of plants tested from the cultivar time series in Experiment 1. Despite showing the drought-tolerance trait association of HWUE and CBL lines, MMEL exhibited typical summer dormancy characteristics where average SDW of the MMEL was 40% of lower than that of the CBL. Also, RSR was higher in the MMEL than in the CBL, and RGS was poor under the conditions tested. These two results may reflect a residual influence of the Mediterranean germplasm component of the parentage.

Scores from field testing by the company were uncorrelated with glasshouse WUE data but did show a modest agreement with SDW and plant hydration trait data. It is suggested that summer performance of the cultivar eventually released might be improved if selection from the pool of elite PRG genotypes for cultivar development included glasshouse-measured WUE as a second selection criterion (or OP as a proxy), in addition to the field score result, assuming that WUE and associated traits are heritable.

Chapter 6

Estimation of quantitative genetic parameters for traits associated with efficient WU in PRG

6.1 Abstract

This chapter describes the evaluation in a fourth glasshouse experiment similar in method to the previous three, of major quantitative genetic parameters of traits contributing to efficient WU and improved summer performance of PRG. Two clonal replicates of five genotypes from 36 PRG half-sib (HS) families and 40 check plants (n=400), carrying the AR37 endophyte, from a germplasm archive were grown in 55 cm tall pots. After 84 days of root development with soil moisture near FC, test plants were well-watered (85–90% FC) for another 21 days, a measurement (M1) of multiple traits conducted, then plants were subjected to a simulated drought (55–65% FC) for the next 28 days, and a second measurement (M2) of the same traits together with some additional traits was carried out. The trait measurements repeated at both M1 and M2 included SDW, WUE, RWC, LWP, OP, Pn, and SC and additionally, RDW_T, RDW_D, RSR, PDW, RGS, SMC_T, and SMC_D were taken only at M2. Phenotypic data collected from HS family plants at M2 were subjected to ANOVA and PCA to examine the HS family means and major drought tolerance trait associations related to efficient WU. A plant breeding decision support software tool (DeltaGen v.0.03) was used to evaluate quantitative genetic parameters of trait measurements (genetic variance, σ_n^2; narrow-sense heritability, h_n^2; predicted genetic gain, ΔG_c; genetic correlation, r_A; correlated response to selection, CR) based on a linear mixed model with the restricted maximum likelihood procedure (REML), considering check plants as fixed effects and all other components (i.e. genotypes within families, families, replicates, rows, columns, and their interaction effects) as random effects. Best linear unbiased prediction (BLUP) was also used for estimating random effects of the mixed model. For the 180 HS family genotypes tested under imposed drought, among- and within-HS family differences for most trait measurements were significant at $p < 0.0001$. Evaluation by PCA of trait associations for this population showed similarities with Experiments 1 and 2 but also some differences. A PCA (PCA 6.2) of phenotypic data for the 9 traits included

in PCA 3.1 (Chapter 3) and PCA 4.1 (Chapter 4) produced a PC1 explaining 60.6% of variation and with coefficients indicating a trait association similar to that of PC1 in PCA 3.1 (characterized as "effective use of water") and a PC2 explaining 15.1% of variation and with coefficients largely matching the HWUE trait association (i.e. WUE-SDW-OP-RWC-RDW_D-SMC_D) of PC3 in PCA3.1. In contrast with previous experiments, inclusion of Pn and SC trait data in this PCA (PCA 6.1) reinforced this result. The key traits showed significant among ($p < 0.0001$) and within HSFs ($p < 0.05$) σ_n^2 and h_n^2 ranging from 0.76 to 0.97 at very low CV (5%–18%), together with a strong correlation with SMC_D ($r > 0.6$, $p < 0.05$), which was not subjected to genetic analyses but was identified as a key selection trait for PRG drought tolerance in PCA, under the conditions tested. ΔG_cestimates of heritable traits ranged from 4.60% to 13.13% at an assumed 30% selection pressure (i.e. ΔG_c of WUE = 7.33%). Despite high r_A estimates observed between the key water relations traits ($r_A > 0.7$, $p < 0.05$), CR estimates of those trait pairs were generally lower than that of ΔG_c estimate of each trait from the single-trait selection (i.e. $CR_{SDW\text{-}RDWD}$= 4.29% and ΔG_c of SDW and RDW_D were 6.58% and 9.92%, respectively). These results suggest that the direct selection of a given PRG population for WUE or a proxy trait is more sensible than indirect selection or multi-trait selection. Moreover, WUE measured at M1 (WUE1) displayed higher estimates of h_n^2 and ΔG_cthan that at M2 (WUE2) (*i.e.* h_n^2 and ΔG_c estimates of WUE1, WUE2 were 0.95, 0.89 and 14.43%, 7.53%, respectively), and r_A between WUE1 and WUE2 was 0.6 at $p < 0.05$, suggesting that PRG populations can effectually be selected for WUE before the onset of drought. In contrast with the majority of literature reports, most quantitative genetic parameters estimated for SC and Pn did not differ significantly between and within HS families ($p > 0.05$) and had high CV estimates at M2. However, SC measured at M1 (SC1) was significantly correlated with SDW at both M1 and M2 ($r_A = 0.90$, $p < 0.05$ and $r_A = 0.12$, $p < 0.1$, respectively), indicating the possibility of selecting drought-tolerant PRG genotypes for SC prior to imposing drought. However, future work is needed to screen a larger PRG HS family population for the key drought tolerance traits at both well-watered and drought conditions in field settings.

6.2 Introduction

Genetic improvement programs in livestock and pastoral industries have been the key to the development of the New Zealand's agricultural sector (Lee et al., 2012). With the consequences of changing climate, germplasm screening for drought tolerance has recently received attention in New Zealand PRG breeding programs to minimize seasonal fluctuations in pasture productivity (Cyriac et al., 2018; Lee et al., 2012). Most pasture selection programs have considered phenotypic variation as the morphological selection criteria (i.e. shoot fresh and dry weight, leaf elongation and tiller number) to evaluate drought tolerance attributes of local (Hatier et al., 2014; He et al., 2017; Lee et al., 2012; Matthew et al., 2012) and exotic PRG germplasms (i.e. Mediterranean, Spanish, and North African) (He, 2016; Hussain, 2013; Lee et al., 2012). Some literature has discussed the magnitude of population variation in cool-season forage species for physiological traits, that are associated with agronomic benefits, under non-stressed (Cui et al., 2015; Fè et al., 2015; Kölliker et al., 1999) and stressed conditions (Cui et al., 2015; Jiang & Huang, 2001b; Jiang et al., 2009; Kubik et al., 2001; Liu & Jiang, 2010; Patel et al., 2015; Turner et al., 2012). However, the relative magnitude of genetic variance in phenotypic variation for such traits has seldom been researched or utilized for breeding purposes in studied species under agronomic drought conditions (Mahon, 1983; Ray & Harms, 1994; Reynolds et al., 2001). Therefore, genetic gains in yield of forage species have been inconsistent for years and lagging far behind those of cereal crops (i.e. jn cereal crops, genetic gains range from 0.25 to 0.73% per year) (Casler & Brummer, 2008; Harmer et al., 2016).

Generating breeding populations and estimating the magnitude of genetic variation for traits of interest under stress conditions is an important step towards selecting the most appropriate trait or traits for breeding purposes (Acquaah, 2012; Rutkoski, 2019; Xu et al., 2017). High genetic gain for a given trait can be achieved by taking advantage of the additive genetic variance among- and within half-sib (HS) or full sib (FS) families (Casler & Brummer, 2008; Wilkins & Humphreys, 2003). Application of among- (AHS) and within- (WHS) family selection methods enable the use of different fractions of the total additive genetic variation available in a breeding population (i.e. AHS and WHS account for ¼ and ¾ portions of total additive variation, respectively (Falconer, 1989)). Moreover, the among- and within-

HS family selection method (AWFS) may be conducted for a single key trait (e.g. WUE) or two different traits simultaneously; the primary trait (e.g. WUE or yield) and a secondary trait (e.g. deep rooting behaviour or osmotic adjustment) (Acquaah, 2012). Thus, AFWS is generally better than AFS or WFS methods, as AFWS represents the total additive genetic variation (Acquaah, 2012; Casler & Brummer, 2008). Such information can successfully be utilized in developing pasture species for increased vegetative persistence and dry matter production under stress conditions, which may also improve the efficiency of pasture breeding programs. As an obligate allogamous species with genetic gametophytic self-incompatibility (Cornish et al., 1980), PRG populations may also exhibit greater within- and between-family genetic variation for most traits (Fè et al., 2015; Thorogood, 2003).

Drought tolerance of plants is associated with a combination of morphological, physiological and anatomical traits, which is often reflected by yield compensations under moisture stress (Blum, 2005; Blum, 2009; Ludlow & Muchow, 1990; Lynch, 2011). Thus, yield has long been the most common trait investigated for drought tolerance or persistence together with easily-measurable morphological traits in crop (de Almeida Silva et al., 2012; Fè et al., 2015) and pasture (Easton et al., 2001; Stewart, 2006) breeding programs. Less attention has been paid to genetic variation for physiological traits that basically determine the yield of studied species under stress conditions (Arab et al., 2019; Des Marais et al., 2013; Kang et al., 2004). Moreover, such information is sparse in selection experiments conducted for temperate forage species including PRG (Cui et al., 2015). In particular, the quantitative genetic information useful for pasture breeding purposes such as additive genetic variation for traits that are directly associated with both pasture production and survival under drought (i.e. WUE) is almost absent from such studies (Attia et al., 2015; Blum, 2005; Blum, 2009; de Almeida Silva et al., 2012; Gresset et al., 2014; Tuberosa, 2012; Ullaha et al., 2019). Nevertheless, genetic variation for WUE at the whole-plant level was inferred through its leaf-level proxy measures such as carbon isotope discrimination or intrinsic WUE under imposed drought in several crop selection experiments (Chen et al., 2011; Ehdaie et al., 1991; Farquhar & Richards, 1984; Gresset et al., 2014; Moghaddam et al., 2013; Rebetzke et al., 2002; Werner & Schnyder, 2012). Thus, there is a need for research that quantifies the significance and magnitude of quantitative genetic parameters including additive

genetic variance and heritability estimates of actual WUE or its proxies and associated traits for forage breeding purposes. Moreover, knowledge of predicted genetic gain and genetic correlations between WUE or its proxy measures and associated traits is imperative when setting breeding objectives in pasture improvement programs (Acquaah, 2012; Grafius, 1978; Machado et al., 2017; Rebetzke et al., 2002). Consequently, such information will enable breeders to choose between direct and indirect selection on alternate traits and methods, particularly when there is a resource limitation associated with the selected trait measurements (Acquaah, 2012; Condon et al., 2002; de Almeida Silva et al., 2012; Johnson & Asay, 1993; Machado et al., 2017). For that reason, representative PRG leaf samples from this experiment were analyzed for carbon isotope discrimination ($\Delta^{13}C$) to address the research question 'Are proxy measures of agronomic WUE (i.e. instantaneous WUE and $\Delta^{13}C$-based intrinsic WUE) fully representative of the actual measurement of WUE in PRG? (data are not shown in this Chapter).

This chapter is focused on estimating quantitative genetic parameters for WUE and associated traits of a key drought-response pattern in previous experiments. Experiment 4 (summer 2018/19), the last experiment of a series of four glasshouse pot experiments conducted throughout 2017/19, was carried out using two copies of five genotypes from 36 PRG HS families and 40 control plants or check plants (n=400) of an advanced PRG breeding population. The water relations and morpho-physiological traits measured under well-watered (M1) and drought (M2) conditions in the same growth cycle of this experiment were subjected to focused statistical analyses similar to those in 'Experiment 1' and quantitative genetic analyses as described in Section 6.3.5 below. Thus, this chapter provides details including methodology, results and discussion and, conclusions of the current experiment that comprises three sub sections. Section 1 characterizes the key trait response pattern of PRG drought tolerance and discusses phenotypic variation observed for measured traits among 36 PRG HS families at two measurement phases, and Sections 2 and 3 discuss the quantitative genetic parameters of the measured traits as evaluated for trait data obtained during both measurement phases. Both sections are aimed at establishing selection criteria for breeding PRG for improved WUE and drought tolerance. Section 3 specifically explores the potential of selecting PRG genotypes for drought tolerance traits prior to the onset of drought.

6.3 Materials and methods

6.3.1 Generation of the plant material; PRG HS families

A random sample of 38 PRG HS families, carrying AR37 endophyte, was taken from a breeding population of 113 HS families improved by a commercial seed company in New Zealand. Of the selected HS families, 36 were used for the estimation of quantitative genetic parameters of measured traits and clonal replicates of two were used as control (check) plants for statistical checking purposes as described below.

Source plants were generated from an isolated poly-cross based on a random sample of 113 plants from an advanced PRG breeding population, designated as Pop II by the company (Faville et al., 2018; Gagic et al., 2018). Seeds from each plant were collectively harvested by hand to constitute an individual HS family. This advanced HS family population was originally derived from crosses between a mid-season-flowering New Zealand cultivar and Spanish ecotypes that successively underwent recurrent selection for vigour, disease tolerance, and endophyte transmission (Gagic et al., 2018).

6.3.2 Glasshouse experiment

This study was conducted as a pot experiment under glasshouse conditions at the Plant Growth Unit, Massey University, New Zealand (40.3709° S, 175.6303° E, 35m amsl), during summer–early autumn, from October 2018 to March 2019. Pots were prepared as described in Section 3.3.2 for Experiment 1.

Eight seeds from each of the 38 HS families were germinated and grown for eight weeks to a size of ≥10 tillers to facilitate division of plants into clonal replicates of four to five tillers each. For 36 HS families, five genotypes were split into two clonal replicates (180 genotypes and 360 plants in total). For two HS families (check plants), five genotypes were split into 4 clonal replicates to provide 20 plants from each HS family, to be used for statistical checking for positional effects on plant growth within the glasshouse. The two clonal replicates of the 180 test genotypes and the 40 statistical check plants were then transplanted into the experimental pots and arranged in a randomised complete block design in the glasshouse together with border plants (Figure 6.1). This specific row-column design (a four block-replicate

set up) was specifically included to check for spatial effects in the glasshouse in Experiment 4 (Figures 6.1 and 6.A1).

Pots were maintained at 85–90% FC (near FC) for 84 days to allow tillering up to ≥20 tillers/pot and for root development. Plants were then defoliated to 5 cm above the soil surface and maintained near FC for another 21 days (M1). Plants were defoliated again and subjected to a drought treatment for 28 days during the subsequent 'measurement' regrowth (M2) as described in Section 6.3.3 below.

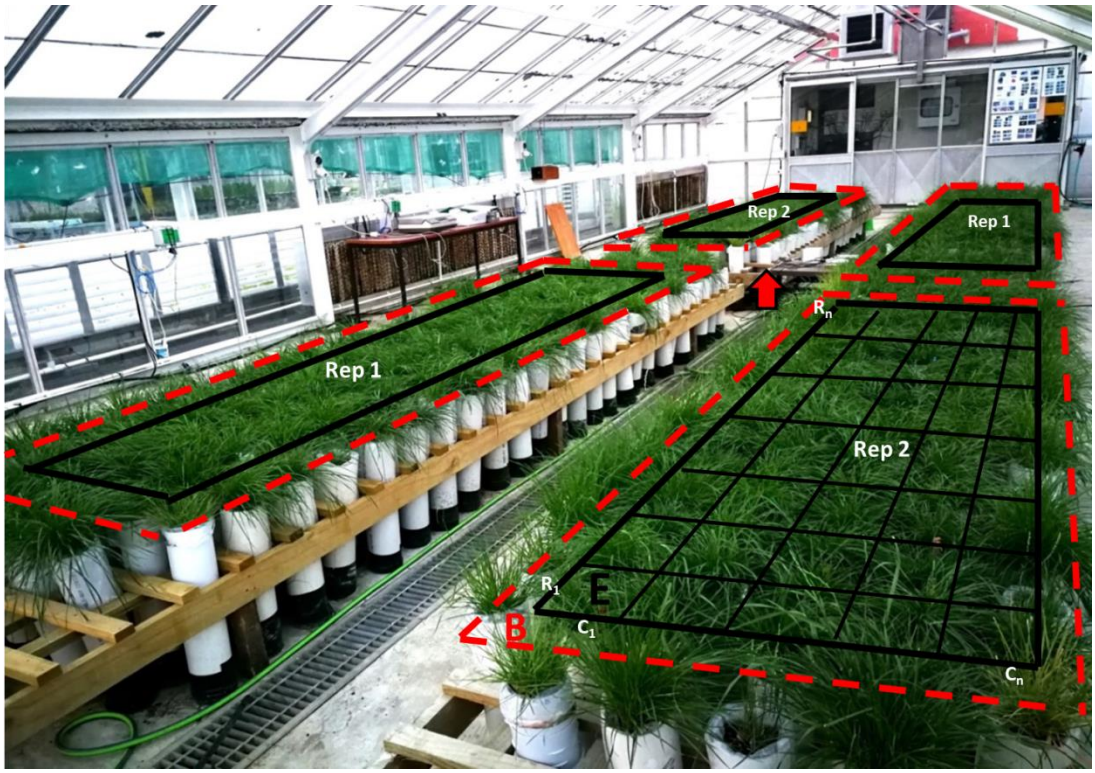

Figure 6.1 General view of the pot arrangement in Experiment 4 before imposing drought stress and 60 days after plant establishment according to Figure 6.2. Abbreviations: Rep, replicate; R_1…R_n, Rows; C_1….C_n, Columns; E, Randomised experimental genotypes from 36 HS family plants; B, Border plants (Note: The arrow in the figure indicates the weather data collection unit located at the canopy level inside the glasshouse; Please see Figure 6.A1 for a detailed illustration).

6.3.3 Drought treatment

During the experiment, the average daily maximum and minimum temperatures inside the glasshouse were 34°C and 16°C, respectively. The relative humidity ranged from 55–85% and light intensity from 800–1000 µmol/m^2/s at the canopy level at midday. The experiment was started in mid-Jan 2019. After a root establishment period of 84 days in fertilised (as first described in Section 3.3.3) 'A; horizon of Egmont Black Loam soil near FC, defoliated genotypes were maintained at a similar soil moisture level (85–95% FC) for another 21 days before the second defoliation (M1). Then, watering interval was progressively increased (together with decreased water volume) until the soil reached 55–65% of FC (MW/MS ~ 35%) (Figure 6.2), as determined by weighing the pots individually on an electronic balance to 1 g precision. Water was added twice weekly as required to keep the pots at constant weight and WU of each plant was recorded. As noted in Chapter 3, 4, and 5, samples of the soil used for filling pots, when tested on a pressure plate apparatus at –0.01, –0.1, and –1.5 MPa had gravimetric soil moisture of 66, 46, and 36%, respectively. Defoliated HS family genotypes from the second defoliation were maintained under imposed soil drought until LWP of randomly sampled plants reached –0.9 MPa which occurred after approximately 28 days. Two sets of detailed measurements of plant water relations and yield traits were carried out within four days; during M1 and M2 phases, for all the genotypes as described below in Section 6.3.4 and illustrated in Figure 6.2. After the third defoliation, irrigation was withdrawn and post-cutting regrowth was recorded after seven days.

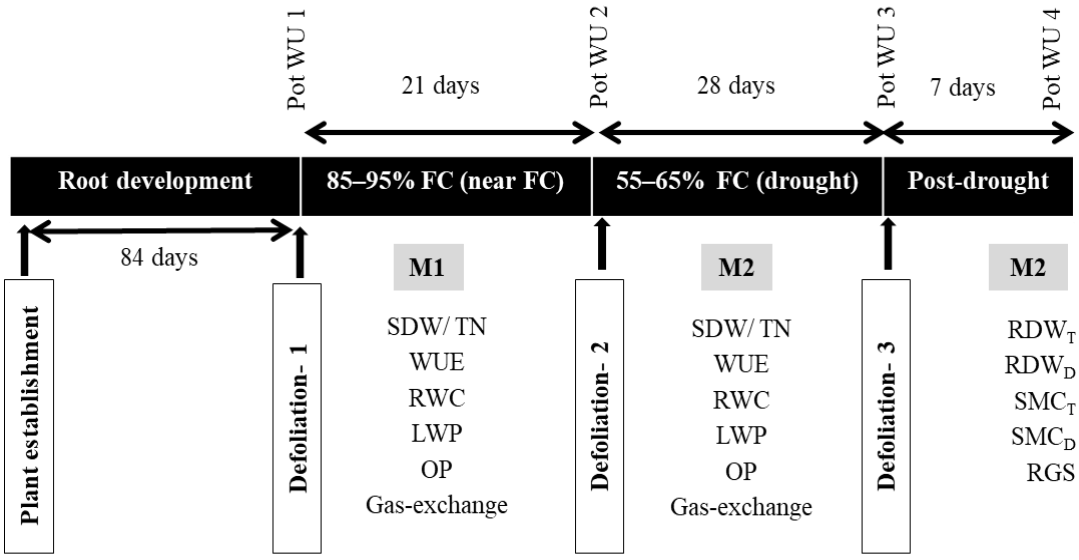

Figure 6.2 Schematic plan of operations for Experiment 4 (Abbreviations: FC, Field capacity; WU, WU; SDW, Shoot dry weight; TN, Tiller number; WUE, Water-use efficiency; RWC, Leaf relative Water Content; LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RDW_T, Root dry weight at 0–20 cm soil depth; RDW_D, Root dry weight at 20–50 cm soil depth; SMC_T, Gravimetric soil moisture content at 0–20 cm soil depth; SMC_D, Gravimetric soil moisture content at 20–50 cm soil depth; RGS, Post-cutting regrowth; M1 and M2, Measurements 1 and 2, respectively).

6.3.4 Measurements and analyses

6.3.4.1 Morpho-physiological traits related to plant water relations

Leaf water relations measurements (i.e. LWP, RWC, and OP), SDW, TN, RGS, pot WU, and WUE were recorded in the last four days of both M1 and M2 phases as described in Chapter 3.3.3 and Chapter 4.3.3 above. Then, watering was completely withdrawn and HS family genotypes were defoliated leaving a 5 cm of stubble to facilitate post-cutting regrowth for seven days and regrowth was visually observed and scored as described in Section 3.3.3.

After RGS was recorded after seven days from the end of M2, root dry weights (i.e. RDW_T and RDW_D) and soil moisture content (i.e. SMC_T and SMC_D) were also measured as described in Chapter 3.3.3 and Chapter 4.3.3 above.

6.3.4.2 Gas exchange measurements

Two major gas exchange variables, Pn and SC were measured using two or three fully expanded young leaves of one representative tillers of each replicate of five genotypes from 36 HS families under artificial lighting, saturating photon flux density (1000 µmol/m^2/s) at an ambient CO_2 concentration of 400 ppm using two portable photosynthesis meters at the same time operated by two persons (LICOR 6400XT, LICOR Biosciences, Inc., Lincoln, NE, USA). Measurements were performed from 10.00 am to 12.00 noon and 2.00 to 4.00 pm for four consecutive sunny days at the end of both M1 and M2. Gas exchange data were recalculated according to the actual total leaf area fitted into the circle shape leaf chamber (diameter: 3 cm): $\sum^{n=2 \text{ or } 3}$ (3 cm × (leaf width)). Temperature of the leaf chamber was around 26°C, and the relative humidity (RH) in the chamber was controlled within a range of 55–65%, as in the glasshouse.

6.3.5 Statistical analyses

Phenotypic analyses

Focused phenotypic analyses were carried out in Minitab version14 and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) software. The ANOVA model of Proc GLM was performed to examine HS family means of traits measured at each/ both M1 and M2. HS family means were generated using the Least Squares Means procedure at M1 and M2. A specified repeat measures model in ANOVA was performed for the trait data that were repeatedly measured at both M1 and M2 to analyse their interaction effects between two measurement stages (MS) at both the genotype level (M1/M2 × Gen) and HS family level (M1/M2 × HSF).

Principal component analysis (PCA) was compiled using 180 genotype means of two clonal replicates of 360 test genotypes (excluding data from check plants) for each trait, to establish key drought-response patterns of the tested HS family population at M2.

Genetic analyses

Analysis of variance was carried out to estimate the magnitude of additive genetic variance among the 36 HS families for the traits measured. The Residual Maximum Likelihood (REML) procedure using a complete random liner model (Eq.1) was used to generate Best Linear Unbiased Predictor (BLUP) values in all the quantitative genetic analyses (White & Hodge, 2013) performed for the measured traits in DeltaGen (v. 0.03) (Jahufer & Luo, 2018).

$$Y_{ijklmn} = M + f_i + s_{ij} + b_k + (fb)_{ik} + r_{kl} + c_{km} + \varepsilon_{ijklmn} \quad \text{(Eq.1)}$$

Where, Y_{ijklmn} is the value of an attribute measured from sample j in half-sib family i in row l and column m of replicate k and i=1,...,n_f, j=1,…,n_s, k=1,...,n_b, l=1,...,n_r, m=1,...,n_c, where f, s, b, r and c are half-sib families, samples, replicates, rows and columns, respectively; M is the overall mean; f_i is the random effect of half-sib family i, N(0,σ^2_f); s_{ij} is the random effect of sample j in family i, N(0,$\sigma^2_{f/s}$); b_k is the random effect of replicate k, N(0,σ^2_b); $(fb)_{ik}$ is the random effect of the interaction between family i and replicate k, N(0, σ^2_{fb}); r_{kl} is the random effect of row l within replicate k, N(0,σ^2_r); c_{km} is the random effect of column m within replicate k, N(0,σ^2_c); ε_{ijklmn} is the residual effect of sample j in half-sib family i in row l and column m of replicate k, N(0,σ^2_ε). Note: Analysis of variance among the HS families (σ^2_f) of the tested HSF population provides an estimate of 1/4 of the total additive genetic variation (σ^2_A) (Acquaah, 2012; Falconer, 1960).

The narrow-sense heritability h^2_n of each trait was estimated on a family mean basis across replicates using DeltaGen software, based on Eq.2;

$$h^2_n = \frac{\sigma^2_A}{\sigma^2_A + \frac{\sigma^2_{Ab}}{n_b} + \frac{\sigma^2_\varepsilon}{n_s n_b}} \quad \text{(Eq.2)}$$

Where, σ^2_A; additive genetic variance (1/4 σ^2_A as estimated among HS families), σ^2_{Ab}, additive-by-replicate interaction and σ^2_ε, experimental error; n_b, number of replicates, n_s, number of samples.

Genetic gain per cycle of selection (ΔG_c), based on among HS family selection, was predicted using DeltaGen based on Eq. 3;

$$\Delta G_c = k_f c \frac{\frac{1}{4}\sigma_A^2}{\sigma_{PF}} \qquad \text{(Eq.3)}$$

Where, ΔG_c, predicted genetic gain using among HS family selection; σ_A^2, additive genetic variance; σ_{PF}, among HS family phenotypic standard deviation; k_f, among HS family selection intensity; c, parental control (0.5) (Assumption: the tested PRG HS family population was a random sample from a breeding population that is currently going through recurrent selection for similar traits) (Faville et al., 2018; Gagic et al., 2018).

The multivariate analysis of variance (MANOVA) option in DeltaGen software that generates a variance and covariance matrix was used to estimate genetic correlation (r_A) between trait pairs of an identified key drought-response trait association (Eq.4);

$$r_A = \frac{COV_{XY}}{\sqrt{\sigma_{A_x}^2 \sigma_{A_y}^2}} \qquad \text{(Eq.4)}$$

Where COV_{XY}, additive genetic covariance between traits X and Y, $\sigma_{A_x}^2$, the additive genetic variance of trait X and, $\sigma_{A_y}^2$, additive genetic variance of trait Y.

Data pattern analysis was conducted to provide a graphical summary of the 36 HS family-by-trait data set. Variance component analysis at individual trait levels generated a HS family-by-trait BLUP (Best Linear Unbiased Prediction) for which the adjusted mean matrix was compiled using a combination of cluster analysis and a PCA using set commands in DeltaGen (Gabriel, 1971; Kroonenberg, 1994)

In DeltaGen, analysis of the primary traits with a secondary trait provides an opportunity to simultaneously estimate h_n^2 for each trait, and their r_A values. These outputs are then automatically integrated into the breeding strategy simulation models in DeltaGen for the estimation of Correlated Response to Selection (CR) of the primary trait based on secondary trait selection. The correlated response (CR_y) to selection in the primary trait (y) for a secondary trait (x) is given by;

$$CR_y = i_x h_x h_y r_A \sqrt{V_{py}}$$

Where i_x is the selection intensity of the secondary trait, h_x and h_y are square roots of the heritabilities of the two respective traits, r_A is the genetic correlation between traits, and $\sqrt{V_{py}}$ is the square root of the phenotypic variance of the primary trait (Acquaah, 2012).

In this experiment, applying a selection pressure of 30% to 36 HS families would result in roughly 11 parents, which was considered better than 10% selection pressure (the usual practice of pasture breeders) that would have selected only four parents. (Note: Increasing selection pressure generally increases ΔG_c of the target traits under selection, depending on their magnitude of σ^2_A in a given population. Also, that increases the number of individuals to be selected from the source populations and thereby the number of parents available to produce the next generation. On the other hand, in a cross pollinating species like PRG, the smaller the number of parents the higher the probability of inbreeding occurrences together with the negative effects of inbreeding depression. Therefore, when deciding on a particular the selection pressure to be applied to a population of individuals, the resulting parent number should also be considered. For example, for a population of 100 individuals, selection pressures of 30%, 20% 10% and 5% will result in parent numbers of 30, 20, 10 and 5, respectively).

6.4 Results

6.4.1 Phenotypic variation for measured traits

For 360 genotypes (36 HS families × 5 genotypes × 2 replicates) evaluated at each consecutive measurement stage (M1 and M2), among- and within-HS family variations for most trait measurements were significant at $p < 0.0001$. However, among- and within-HS family variance estimates recorded for OP and RWC measured at M1 and those for gas exchange measures taken at M2 were less significant at $p > 0.05$ (Table 6.1).

Table 6.1 Overview of data: Half-sib family (HSF) population means (Pop mean) with range and standard error of trait means (±SEM) and their statistical probabilities (p) for replicates (Rep), HSFs, genotypes-within-HSF population (Gen), and genotypes-within-HSF (Gen [HSF]) effects of measured traits at each consecutive measurement phase (MS; M1 and M2) and 'p' of MS and interaction effects between MS and HSF and MS and Gen for the measurements repeated at both M1 and M2.

	MS	Trait[1]	Pop mean (range)	SEM	p(Rep)	p(HSF)	p(Gen)	P(Gen[HSF])	p(MS)	p (MS×HSF)	p (MS×Gen)
Repeat measures	**M1[2]**	**SDW (g/plant)**	4.11 (0.80–7.58)	0.47	ns	<0.0001	0.0022	0.022	<0.0001		
		WUE (g WU/g DM)	609.50 (254.70–2670.00)	74.00	ns	<0.0001	0.0039	ns	<0.0001		
		RWC (%)	86.46 (74.09–99.59)	0.97	ns	ns	ns	0.0860	<0.0001		
		LWP (MPa)	–0.39 (–0.23 to –0.77)	0.44	ns	<0.0001	0.0530	0.0007	<0.0001		
		OP (MPa)	–1.45 (–0.78 to –2.30)	0.65	ns	ns	ns	0.0443	<0.0001		
		Pn (µmol/m^2/s)	16.12 (5.75–37.70)	0.28	ns	<0.0001	0.0092	0.0196	<0.0001		
		SC (mmol/m^2/s)	0.20 (0.042–0.56)	0.005	ns	<0.0001	0.0085	0.0135	<0.0001		
	M2 (D)[3]	**SDW**	3.76 (1.5–5.4)	0.33	ns	<0.0001	<0.0001	<0.0001	<0.0001	ns	<0.0001
		WUE	492.02 (193.96–986.59)	73.71	ns	<0.0001	<0.0001	<0.0001	<0.0001	ns	<0.0001
		RWC	55.52 (45.83–72.76)	6.15	ns	<0.0001	0.0010	<0.0001	<0.0001	0.0067	0.0286
		LWP	–0.74 (–0.62 to –1.23)	0.38	ns	<0.0001	<0.0001	<0.0001	<0.0001	ns	<0.0001
		OP	–2.45 (–1.74 to –3.53)	2.58	ns	<0.0001	<0.0001	<0.0001	<0.0001	ns	<0.0001
		Pn	5.07 (1.82–11.86)	2.15	ns	0.0178	ns	0.0928	<0.0001	ns	<0.0001
		SC	0.06 (0.01–0.22)	0.03	ns	ns	ns	ns	<0.0001	ns	<0.0001
	M2 (PD)[3]	**RDW_T (g/plant)**	1.59 (0.61–2.99)	0.20	ns	<0.0001	<0.0001	<0.0001			
		RDW_D (g/plant)	0.45 (0.02–0.94)	0.08	ns	<0.0001	<0.0001	<0.0001			
		RDW_{Tot} (g/plant)	2.05 (0.70–3.73)	0.23	ns	<0.0001	<0.0001	<0.0001			
		PDW (g/plant)	10.10 (4.88–14.50)	0.54	ns	<0.0001	<0.0001	<0.0001			
		RSR (%)	25.70 (10.39–68.31)	3.37	ns	<0.0001	<0.0001	<0.0001			
		RGS	3.08 (0–5)	0.56	ns	<0.0001	0.0039	<0.0001			
		SMC_T (%, w/w)	23.05 (18.37–40.84)	2.71	ns	<0.0001	ns	<0.0001			
		SMC_D (%, w/w)	26.75 (20.09–44.62)	3.56	ns	<0.0001	ns	<0.0001			

[1]Trait abbreviations: SDW, Shoot dry weight; WUE, Water-use efficiency; RDW_T, Root dry weight at 0–20cm depth; RDW_D, Root dry weight at 20–50cm depth; RDW_{Tot}, Total root dry weight; RSR, Percentage total root: total shoot ratio; PDW, Total plant dry weight; SMC_T, Gravimetric soil moisture content at 0–20 cm depth; SMC_D, Gravimetric soil moisture content at 20–50 cm depth; LWP, Predawn Leaf water potential; OP, Osmotic potential; RWC, Leaf relative water content; Pn, Photosynthesis; SC, Stomatal conductance; MS, Measurement stage; [2]M1 and M2, Measurement phases 1 and 2; [3]D, Drought; PD, Post-drought measurements (Note: 'ns' denotes non-significance at 95% probability level, the range of five genotypes-within-HS families of each trait appears in parentheses, and dark cells represent data features of the repeated traits in both measurement phases).

Trait measurements that were repeated at both M1 and M2 significantly differed between the two measurement phases ($p < 0.05$; Table 6.1). For example, WUE showed a 4-fold difference (ranging from 250 to 1000 g water per g DM harvested) at M1 (i.e. 85–90%FC) and a 10-fold difference (ranging from 250 to 2600 water per g DM harvested) and at M2 ($p < 0.0001$). In the output of the specified repeat measures model (Table 6.1), the interaction effects between two measurement stages (MS) and the measurements taken in each MS at the genotype level (i.e. M1/M2 × Gen) were significant for all repeated traits while that at the HS family level (i.e. M1/M2 × HSF) were significant only for RWC trait at $p < 0.05$ (Table 6.1).

Two PCAs were compiled for the trait data of this experiment (Table 6.2). PCA6.1 included a total of 15 morpho-physiological traits, while PCA6.2 included morphological traits, which are related to PRG water relations, as previously carried out in Experiments 1, 2, and, 3. These PCAs narrowed down wider data dimensions and the large data variation into several independent associations (PCs) of correlated traits (PC coefficients) allowing the extraction of independent biologically driven trait associations from each PC. From PCA6.1, the first 5 PCs explaining 95% of data variation and from PCA6.2, the first 3 PCs explaining 88% of the total variation, (eigenvalue > 0.7 (Jolliffe, 2003)) were selected for presentation from the total of 15 and 9 PCs, respectively. In PCA6.1, PCs 1 to 5 explained 53%, 16%, 12%, 6%, and 4% of data variation, respectively. PC1 identified variation among individual genotypes based on their low WU or high WUE, improved plant hydration (i.e. positive PC coefficient for RWC and negative coefficients for OP and LWP) and high dry matter allocation to both shoots and roots (i.e. positive PC coefficients for SDW, RDW_T, and RDW_D) with high net assimilation (i.e. positive PC coefficient for Pn) at the expense of soil moisture (i.e. negative coefficient for SMC_D). The second PC explicated a drought-response pattern characterized by large plant size, less WU, high tissue hydration, high net assimilation, high dry matter allocation for deep roots (high RDW_D), and less depletion of SMC (high SMC_D), with a negative factor loading for SC. The third PC described a pattern of diversity among genotypes for plant dry matter allocation to shoots and roots (i.e. high SDW linked to low RSR) and also reduced plant hydration and gas exchange. The fourth PC described a pattern of variation among genotypes due to differing dry matter allocation to roots and PRG WUE. The fifth PC differentiated less hydrated large-

sized genotypes (Table 6.2). It was salient to note that PC1 and PC2 of PCA6.1 were highly comparable to that of PCA6.2 (i.e. correlation coefficients between PC scores of the first five PCs from PCA6.1 and 6.2 are presented in Table 6.A1). In particular, PC2 of both PCAs explicated variation based on individual genotypes for the key drought-response trait association that contributed more to high WUE in Experiment 1, 2, and 3 above (Tables 3.1, 4.1, and 5.1, respectively).

Table 6.2 Principal component (PC) coefficients for the first five and three PCs generated by PCA6.1 and PCA6.2 of 17 and 9 morpho-physiological and water relations data, respectively, as obtained from 180 genotype means of two clonal replicates of 360 test PRG genotypes (excluding check plants) of the HS family population, measured at M2.

	PCA6.1					PCA6.2		
	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC1**	**PC2**	**PC3**
Eigen value	8.98	2.63	2.11	1.01	0.69	5.45	1.36	1.07
% variation explained	52.8	15.5	12.4	6.0	4.0	60.6	15.1	11.9
Cumulative % variance	52.8	68.3	80.7	90.7	94.5	60.6	75.7	87.6
SDW	0.216	0.201	0.411	-	0.251	0.299	0.343	0.536
WUE	–0.234	–0.168	–0.399	0.176	–0.163	–0.319	–0.315	–0.531
RWC	0.158	0.436	–0.278	-	–0.238	0.206	0.500	–0.572
LWP	0.283	–0.188	-	-	–0.204	0.353	–0.379	-
OP	–0.226	–0.380	-	-	0.223	–0.309	–0.450	0.286
RDW_{Tot}	0.250	–0.216	–0.196	–0.406	0.272			
RDW_D	0.282	0.301	-	0.428	0.283	0.357	0.168	–0.159
RDW_T	0.289	–0.178	–0.174	–0.137	0.306	0.355	–0.283	–0.158
PDW	0.295	-	0.209	–0.145	0.300			
RSR	0.105	–0.297	–0.530	-	0.145			
SMC_T	–0.276	-	-	0.213	0.323			
SMC_D	–0.297	0.102	-	-	0.287	–0.383	0.183	-
RGS	0.300	0.121	-	-	–0.243	0.382	–0.213	-
Pn	0.135	0.464	–0.301	–0.117	-			
SC	-	–0.433	–0.301	-	0.287			

Trait abbreviations: SDW, Shoot dry weight; WUE, Water-use efficiency (g WU/g SDW); RDW_T, Root dry weight at 0–20cm depth; RDW_D, Root dry weight at 20–50cm depth; RDW_{Tot}, Total root dry weight; RSR, Percentage total root: total shoot ratio; PDW, Total plant dry weight; SMC_T, Gravimetric soil moisture content at 0–20 cm depth; SMC_D, Gravimetric soil moisture content at 20–50 cm depth; LWP, Predawn Leaf water potential; OP, Osmotic potential; RWC, Leaf relative water content; Pn, Photosynthesis; SC, Stomatal conductance.

6.4.2 Quantitative genetic parameters of trait measurements

Among the fifteen plant traits measured, most traits showed significant genetic variance within HS families or HSFs ($\sigma^2_{f/s}$) at $p < 0.05$. Genetic variation among-HSFs (σ^2_f) was statistically significant for the traits; SDW, WUE, RDW_D, PDW, RGS, OP, and LWP (Table 6.3). Other variance components (i.e. replicate effect, σ^2_b; row effect, σ^2_r; column effect, σ^2_c; HSF × replicate effect, σ^2_{fb}; HSF × column effect, σ^2_{fc}; HSF × row effect, σ^2_{fr}) were less significant for all traits measured in the current experiment ($p > 0.03$). Narrow-sense heritability estimates (h^2_n) of most traits were high (i.e. $h^2_n > 0.8$; Table 6.3) except for RSR, RWC, Pn, and SC in the HS family population when evaluated at 55–65% FC (M2). The coefficient of variation of all heritable plant traits varied from 5% to 18%.

High estimates of predicted genetic gain (ΔG_c) were noted for RGS, RDW_D, WUE, and SDW traits (13.13%, 9.92%, 7.33%, and 6.58%, respectively) while those of other trait measurements were moderate (ranging from 3% to 6%) at the 30% selection intensity (Table 6.4)

Genetic correlation coefficients were significant for most trait combinations of WUE at $p < 0.05$ (Table 6.4). In particular, trait associations between WUE, OP, LWP, RDW_D, RGS, and SDW or PDW traits exhibited genetic correlations above 0.7 at $p < 0.05$. Pearson correlation estimates were also computed for all measured traits including SMC_D, for which heritability cannot directly be estimated although it is an important drought tolerance trait or a proxy trait of RDW_D, was not included in quantitative genetic analysis in this experiment deserved extra attention. Accordingly, it was found that this trait had strong correlations with most traits (i.e. $p < 0.05$; Table 6.A2) measured at M2, similar to that observed in Experiment 1, 2, and 3 (Chapters 3, 4, and 5).

Table 6.3 Narrow-sense heritability (h_n^2), significant variance components with their standard errors (±); genotype-within-HS families ($\sigma^2_{f/s}$) and among-HS families ($\sigma^2_{f/s}$) and residual error (σ^2_ε) and coefficient of variation (CV%) of measured plant traits as estimated among the 36 HS families at M2.

Trait	h_n^2	Variance component			CV%
		$\sigma^2_{f/s}$	σ^2_f	σ^2_ε	
SDW	0.95±0.017	0.28±0.04	0.15±0.05	0.08	7.52
WUE	0.89±0.032	4691±847	3928±1293	4603	13.77
RWC	ns	15±4.54	ns	39.00	-
LWP	0.95±0.017	0.82±0.10	0.30±0.11	0.14	5.09
OP	0.76±0.096	9±1.46	2±1.12	7.11	10.47
RDW_T	0.81±0.13	0.16±0.02	ns	0.04	12.18
RDW_D	0.90±0.041	0.03±0.004	0.007±0.003	0.007	18.08
RDW_{Tot}	0.89±0.055	0.27±0.03	ns	0.05	10.86
PDW	0.97±0.007	2±0.21	1±0.30	0.21	4.48
RSR	ns	47±6.10	ns	11.10	-
RGS	0.94±0.016	0.47±0.07	0.52±0.15	0.32	18.19
SC	ns	ns	ns	0.01	-
Pn	ns	0.30±0.34	ns	4.45	-

Trait abbreviations: SDW, Shoot dry weight (g/plant); WUE, Water-use efficiency (g WU/g SDW); RDW_T, Root dry weight at 0–20cm depth (g/plant); RDW_D, Root dry weight at 20–50cm depth (g/plant); RDW_{Tot}, Total root dry weight (g/ plant); RSR, Percentage total root: total shoot ratio; PDW, Total plant dry weight (g/plant); LWP, Predawn Leaf water potential (MPa); OP, Osmotic potential (MPa); RWC, Leaf relative water content (%); Pn, Photosynthesis (µmol/m^2/s); SC, Stomatal conductance (mmol/m^2/**s**) (Note: Less significant standard errors of variance components in the mixed-effects model of this analysis reflects on the high significance in the contribution of random effects to the model and the precision of the model itself). (Note: 'ns' denotes not statistically significant).

Table 6.4 Genetic correlation coefficients between heritable plant trait pairs ($p < 0.05$) and predicted genetic gain (ΔG_c) of each trait by single-trait selection at 30% of selection pressure in the tested perennial ryegrass HS family plant population under imposed drought.

Trait	SDW	WUE	RDW_T	RDW_D	RDW_{Tot}	PDW	RGS	OP	LWP
ΔG_c (%)	6.58	7.33	4.93	9.92	6.08	6.00	13.13	3.30	4.26
SDW									
WUE	–0.94								
RDW_T	0.25	–0.38							
RDW_D	0.63	–0.63	0.55						
RDW_{Tot}	0.44	–0.53	0.95	0.8					
PDW	0.78	–0.86	0.76	0.76	0.85				
RGS	0.45	–0.54	0.81	0.72	0.88	0.80			
OP	–0.83	0.82	–0.55	–0.81	–0.72	–0.82	–0.72		
LWP	–0.43	0.48	–0.65	–0.77	–0.78	–0.73	–0.68	–0.87	

Trait abbreviations: SDW, Shoot dry weight (g/plant); WUE, Water-use efficiency (g WU/g SDW); RDW_T, Root dry weight at 0–20cm depth (g/plant); RDW_D, Root dry weight at 20–50cm depth (g/plant); RDW_{Tot}, Total root dry weight (g/plant); PDW, Total plant dry weight (g/plant); LWP, Predawn Leaf water potential (MPa); OP, Osmotic potential (MPa) (Note: Phenotypic correlations between all measured traits at M2 are presented in Table 6.A2).

Significant genetic correlation estimates that were noted for the trait pairs; WUE-OP and WUE-LWP and as well as OP-LWP (r_A = 0.8, 0.5, and 0.9, respectively) at M2, suggested the importance of comparing correlated response (CR%) estimates of measured traits in the current experiment. For most measured traits, CR% of intra-population selection, as estimated for heritable trait pairs (except for RDW_D-OP and RGS-RDW_D) were generally lower than the ΔG_c of each trait at 30% of selection intensity (Table 6.5). For example, when both SDW and RDW_D traits are selected together, CR was estimated as 4.29% whereas ΔG_c values of those traits from single-trait selection were 6.58 and 9.92, respectively (Table 6.5). Despite the high correlation estimate observed for the OP-LWP trait pair (r_A= 0.87), CR% estimates of OP and each measured trait were higher than that of LWP with most traits (Tables 6.4 and 6.5).

Table 6.5 Correlated response to selection (CR%) estimates of heritable traits when perennial ryegrass genotypes are simultaneously selected for two traits (primary and secondary) within the tested HS family population at 30% of selection intensity under imposed drought. Green, yellow, white, and grey color cells highlight the predicted genetic gain estimates of single trait selection, significant CR estimates of pair-wise trait selection at $p < 0.05$, less important trait pairs ($p > 0.05$) and, significant but spurious trait associations, respectively.

CR%	SDW	WUE	RDW_T	RDW_D	RDW_{Tot}	PDW	RGS	OP	LWP
SDW	6.58								
WUE		7.33							
RDW_T			4.93						
RDW_D	4.29	4.70		9.92					
RDW_{Tot}					6.08				
PDW						6.00			
RGS		4.45		8.08			13.13		
OP	5.23	6.75		7.62			2.55	3.03	
LWP	2.28	2.28		3.70			3.70		4.26

Trait abbreviations: SDW, Shoot dry weight (g/plant); WUE, Water-use efficiency (g WU/g SDW); RDW_T, Root dry weight at 0–20 cm depth (g/plant); RDW_D, Root dry weight at 20–50cm depth (g/plant); RDW_{Tot}, Total root dry weight (g/plant); PDW, Total plant dry weight (g/plant); LWP, Predawn Leaf water potential (MPa); OP, Osmotic potential (MPa).

6.4. Selection of PRG genotypes for major traits prior to the onset of drought

Selected traits (i.e. WUE, SDW, RWC, OP, and LWP) were measured twice consecutively during the experimental period at 'M1 (near FC)' when plants were well-watered (during the 3–4 month period in the plant growth cycle from establishment) and at 'M2 (60% FC)' when plants were in moisture deficit (during 4–5 months period of the plant growth cycle from establishment which was the experimental growth) in the same growth cycle. Consequently, trait correlations between M1 and M2 were compared to explore the possibility of selecting PRG genotypes for the key traits in the tested PRG HS family population before imposing the soil drought. The comparison of trait correlations between M1 and M2 revealed strong correlation estimates for WUE and LWP traits (i.e. $r_A > 0.6$ for WUE1-WUE2 and LWP1-LWP2, respectively; Figure 6.3) at $p < 0.05$. Moreover, WUE and LWP also showed high h_n^2 estimates of each trait at M1 ($h_n^2 > 0.9$). Despite high h_n^2 estimates, ΔG_c estimates of WUE1 and LWP1 were higher than those of WUE2 and LWP2 (i.e. ΔG_c of WUE1, LWP1 and WUE2, LWP2 were 14.43%, 8.13% and 7.54%, 4.26%, respectively). Predicted genetic gain of most measured traits was higher at M1 than that at M2 for most traits except for OP which was not heritable at M1 (Table 6.2). These results suggest the potential of WUE and LWP trait measurements to be used as pre-drought selection criteria targeting PRG drought tolerance. However, further research is warranted for the confirmation of current results before accepting them in a pasture breeding work.

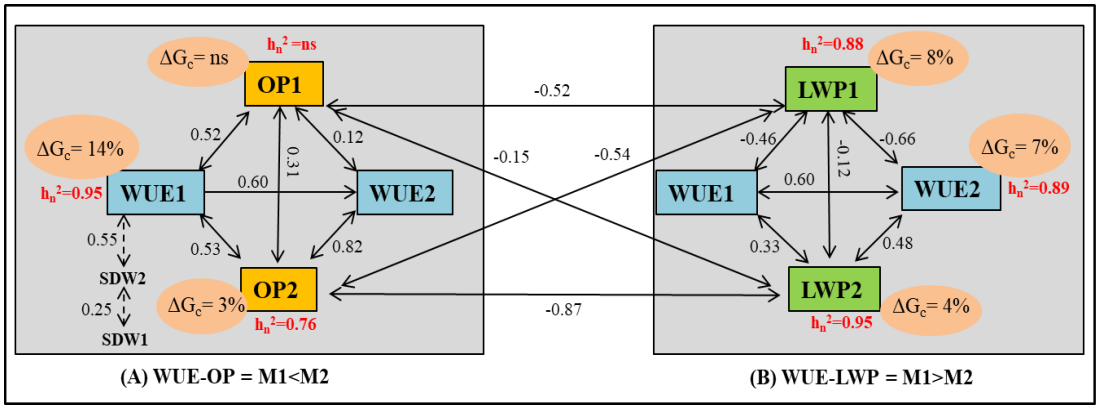

Figure 6.3 A diagram comparing genetic correlations, heritability, and ΔG_c estimates of WUE, OP, and LWP traits within and between two consecutive measurement phases of Experiment 4 (Abbreviations: h_n^2, Narrow-sense heritability; ΔG_c, predicted genetic gain; ns, Non-significant at $p<0.05$; WUE, Water-use efficiency; LWP, Predawn leaf water potential; OP, Osmotic potential). Note: As supportive information, genetic correlations between shoot dry weight (shoot dry weight at M1 and M2: SDW1 and SDW2, respectively) are also presented. Two-headed solid or broken arrows illustrate genetic correlations of trait pairs within and between M1 and M2.

Genetic variance components, h_n^2 estimates, and r_A for 'SC' and 'Pn' representing gas exchange measurements of tested HS families were not significant under imposed drought or at M2 ($p > 0.05$, Table 6.3). Conversely, most quantitative genetic parameters estimated for SC and Pn at near FC or at M1 were significant ($p < 0.05$, Table 6.6). Also, r_A estimates between SC, WUE, SDW, and LWP trait pairs were significant at M1 ($p < 0.05$, Table 6.6). Consequently, at M1, CR% estimate of the SC-SDW trait pair was as high (7.33%) as the ΔG_c estimates of single-trait selection of each trait under tested conditions (i.e. ΔG_c of SC and SDW were 7.82% and 6.87%, respectively). Moreover, SC1 showed a weak but positive correlation with SDW measured at M2 ($r_A= 0.12$; $p < 0.1$), implying its ability to account for pre-drought selection of drought-tolerant PRG genotypes targeting high summer yield (Table 6.6).

Table 6.6 Estimates of genetic (r_A) correlation coefficients, narrow-sense heritability (h_n^2), and predicted genetic gain ($\%\Delta G_c$) of selected traits (i.e. that were recorded as heritable traits at M1), if they were selected from the tested HS family population under an assumed 30% selection pressure (11 HSFs selected from 36 HSFs tested) in both M1 and M2. Correlated response (CR %) estimates of the same traits measured at both measurement stages with one common gas exchange parameter measured at M1 are also presented. OP is included as an addition to the key information presented considering its importance as a surrogate to SC and WUE traits in the tested perennial ryegrass HS family population.

		WUE1	**WUE2**	**SDW1**	**SDW2**	**LWP1**	**LWP2**	**OP1**	**OP2**	**SC2**	**Pn2**	**SC1**	**Pn1**
	h_n^2	0.95	0.89	0.69	0.94	0.88	0.95		0.76	***ns***	***ns***	0.63	0.64
	ΔG_c (%)	14.43	7.33	6.87	6.58	8.13	4.26		3.03			7.82	6.18
SC1	**r_A**	–0.64**	–0.20*	0.92***	0.12*	0.60***	***ns****	–0.75*	–0.17*	***ns****	***ns****		
Pn1		–0.70**	–0.23*	0.90***	0.16**	0.64***	***ns****	–0.79*	–0.20*	***ns****	–0.18*	0.94***	
	CR_{SC1} (%)	6.79	1.62	7.33	0.33	6.84			2.16				

Trait abbreviations: SDW, Shoot dry weight (g/plant); WUE, Water-use efficiency (g WU/g DM); LWP, Predawn Leaf water potential (MPa); OP, Osmotic potential (MPa); Pn, Photosynthesis (µmol/m/s^{-1}); SC, Stomatal conductance (mmol/m^2/s^{-1}) (and 1 and 2 numbers after each trait abbreviation denote the same trait measurement taken at the first (M1) and second (M2) measurement stages, respectively (Note: ***=$p < 0.0001$, **=$p < 0.05$, *=$p < 0.01$; '*ns*' denotes the non-significance at $p>0.05$ of quantitative genetic parameters presented; empty cells represent non-significant traits/ trait associations and thus, their information is not presented; bold letters and numbers with asterisk highlight significant features of data presentation).

6.5 Discussion

6.5.1 The value of quantitative genetic analysis as in this research

It is evident that population improvement is possible whenever there is an adequate variation within the breeding pool for the traits of interest and selection has focused on the right traits to be measured in the right environment (Cooper et al., 2014). It was expected that PRG, as an allogamous species, would generate a considerable genetic variability for the target traits (Aastveit & Aastveit, 1990; Thorogood, 2003) and this was confirmed for most measured traits in the current results (Table 6.1). In search of the major drought tolerance traits in the results of a series of experiments in this research, PCAs that were compiled for the diverse data in each experiment (i.e. Experiment 1, 2, 3, and 4 presented in Chapter 3, 4, 5, and 6, respectively) discovered major drought-response patterns comprising multiple trait associations that elucidate PRG drought tolerance. Among them, a 'WUE-OP trait association' was identified as the key contributor to PRG drought tolerance. It was also found that this key trait association may be expressed in different ways involving modification of root growth in the top soil layer (0–20 cm soil depth) in addition to deep rootedness promoted by more negative OP, which may in turn influence the rate of soil dry down or moisture retention in a drought. For example, PC1 of PCAs 3.1, 3.2 (Chapter 3), 4.2 (Chapter 4), and 6.1 (Chapter 6) indicated that high WUE of PRG may be achieved through improved plant hydration (associated with more negative OP) and high dry matter allocation to both shoots and roots with high Pn at the expense of soil moisture. PC3 of the same PCAs or PC1 of PCAs 3.3 (Chapter 3), 4.1 (Chapter 4), 5.1, 5.2 (Chapter 5), and 6.2 (Chapter 6) indicated a pattern where WUE is linked to large plant size, less WU, high tissue hydration, high Pn but low SC, high dry matter allocation for deep roots, and less depletion of SMC.

Plants achieving WUE, by depleting soil moisture to keep growing as in the first trait association above, align with Blum's recommendation that crop improvement should aim for 'effective use of water' (Blum, 2009). However, plants exhibiting the second trait association where high SDW is achieved while conserving soil moisture demonstrate a true WUE without the negative impact on yield which Blum (2009)

believed might occur. From a PRG breeding perspective, the latter trait association is most likely to assist an optimising summer production in moisture limited environments (and was applied in selection of HWUE genotypes in Experiment 1 in Chapter 3) if it's single trait heritabilities are high enough to facilitate selection with higher genetic gains. Moreover, breeding PRG for the traits related to true WUE may become feasible, if there's an easily-measurable surrogate of WUE, for which one or few secondary traits should be genetically correlated to WUE together with their higher estimates for the correlated response to selection than the genetic gain of the primary trait selection.

In this experiment, a HS family population, which had originated from a proprietary breeding pool and was developed from crosses between genotypes from a mid-season-flowering New Zealand cultivar and Spanish ecotypes, was used to analyse quantitative genetic parameters of measured traits. As this progeny has been subjected to successive cycles of recurrent selection for persistence (Faville et al., 2018; Gagic et al., 2018), it was envisaged that working with it would provide precise quantitative data for measured drought tolerance traits. The intention for this PRG HS family population was to be exposed to a moisture deficit challenge of sufficient duration and intensity to display its genetic potential for efficient WU and drought tolerance. Based on the experience with the earlier series of experiments 1 (Chapter 3), 2 (Chapter 4), and 3 (Chapter 5), an adequate drought challenge was imposed through the maintenance of optimum soil volume: plant DW ratio and high WHC of the test soil in this experiment. Thus, the moisture deficit challenge provided in the current experiment was similar to that of Experiment 1, 2, and 3 and also representative of one drying cycle under field conditions as faced by pasture plants during summer in New Zealand.

6.5.2 The degree of genetic control of drought tolerance traits

6.5.2.1 Genetic variances and heritability estimates of measured traits

Results from the evaluation of two replicates of 180 HS family genotypes and 40 check plants indicated a significant 'among- and within-HS families' phenotypic variation for most traits measured at M2, enabling the selection for the traits of interest (Table 6.1). As noted in Section 6.1 above, phenotypic expression of a particular trait does not

completely reflect its genetic expression because genetic differences are often confounded with environmental variation and GE interaction effects. Even so, in pasture breeding programs, most selection techniques are focused on the variations in the phenotypic expression of the target traits (Cui et al., 2015), and that may well have resulted in slow progress in pasture improvement programs (see Chapter 2). Therefore, it is advisable to estimate genetic variance components and heritability estimates of selected traits in this research before taking PCA results from previous experiments into consideration in PRG breeding programs.

High genetic variance of a selection trait indicates its potential for genetic improvement through selection under imposed drought (Mahon, 1983; Ray & Harms, 1994; Reynolds et al., 2001). In the current results, estimated genetic variation among the 36 HS families under imposed drought was significant for most traits measured at M2 ($p < 0.05$, Table 6.3). In particular, genetic variances of both HS families and genotypes-within-HS families were large for SDW, PDW, WUE, RDW_D, OP, LWP, and RGS traits (Table 6.3), suggesting that both among- and within-HS family selection may effectively be conducted for each selected trait or for a combination of traits (Acquaah, 2012). For example, the use of this approach of the primary trait (i.e. WUE or SDW) and one of the secondary traits (i.e. RDW_D and OP) enables the use of different fractions of the total additive genetic variation available in the tested breeding population (Falconer, 1989). However, the current experiment facilitated only the selection among 5 genotypes in each family of the tested 36 HS families where the sample size of each tested HS family was too small to carry out an effective within-family selection. Therefore, h_n^2 of measured traits was estimated primarily based on the variation among HS families (i.e. mean family basis) which is equal to ¼ of the total additive variation. Even so, estimated h_n^2 values of the major traits were mostly >0.8 (see below). Also, for the traits with higher genetic variances, the CV% ranged from 5% to 18% (Table 6.3) engendering confidence that the estimated genetic variances and h_n^2 estimates were not unduly affected by data noise (Jia, 2017; Nyquist & Baker, 1991).

Narrow-sense heritability values (where significant) for the measured traits ranged from 0.76 to 0.97 in the 36 HS families evaluated at M2 (Table 6.3). More importantly, the

major traits associated with high WUE in the key drought response pattern identified in the previous series of experiments showed strong genetic effects at M2 (i.e. h_n^2 of WUE, SDW, RGS, OP, LWP, and RDW_D were 0.89, 0.94, 0.94, 0.76, 0.95, and 0.90, respectively). Despite the extensive literature available on quantitative genetic parameters for surrogates of WUE (mainly carbon isotope discrimination) in arable crops, there are only a few recently published examples where heritability estimates of actual WUE was discussed for studied plant species or forage species (see Section 2.2.4.1). As mentioned elsewhere, genetic improvement of actual WUE using traditional methods has proven difficult due to its low throughput nature and environmental heterogeneity in field settings. However, the question remains to what extent the genetic information obtained on the proxy measures of actual WUE are applicable in the use of true WUE for breeding purposes. In addition to that research gap, there are only a very limited number of studies that discuss quantitative genetic information on multiple drought tolerance traits that constitute key drought-response patterns. One such example includes a recent study that utilized a high-throughput phenotyping platform to overcome practical limitations of WUE measurements in an interspecific *Setaria italica* × *Setaria viridis* cross. Results of that study found that plant size, plant WU, and WUE are heritable traits and more importantly, WUE was directly linked to soil water availability, so also allowing for a genetic dissection of the components of plant WUE under different watering treatments (Feldman et al., 2018). Thomas (1990) found high broad-sense heritability for OP (0.76) and high repeatability or the significant correlation between more negative leaf OP and RGS in tested PRG genotypes over two drought selection cycles. The current results provide information on a combination of key quantitative genetic parameters of 15 morpho-physiological and water relations traits of a major PRG drought-response trait association based on data obtained in an advanced breeding population under simulated summer drought cycle in New Zealand. Also, in the current results, significant correlation estimates observed between most measured traits and SMC_D, which was not subjected to genetic analyses on the basis it is not a plant measurement but was identified as a key selection trait for PRG drought tolerance with high phenotypic variance among- and within- test germplasms and PCA results with a substantive PC coefficient in previous experiments,

($r > 0.6$, $p < 0.05$; Table 6.A2) indicated the importance of including SMC measurements when selecting PRG genotypes for drought tolerance.

6.5.2.2 Predicted genetic gain of heritable traits

According to Dalal et al. (2017), traits that exhibit high heritability can directly and effectively be selected for plant stress tolerance with high genetic gain. Hence, significant genetic variances and high h_n^2 estimates of the major traits in the current experiment showed the potential for their genetic improvement under conditions tested. ΔG_c estimates of WUE, SDW, RDW_D, OP or LWP, and RGS ranged from moderate to high (i.e. 7.33%, 6.58%, 9.92%, 3.30% or 4.24% and, 13.13%, respectively) at an assumed 30% selection pressure (i.e. 11 out of 36 HS families) based on data of 36 HS families collected at M2 (Table 6.4). However, the consistency of the results under different environmental challenges needs to be elucidated, as G × E effects may confound estimates of the realized genetic gain. Also, ΔG_c may not be fully representative of the realized genetic gain of the target traits under such conditions (Fè et al., 2015; Rutkoski, 2019).

6.5.2.3 Genetic correlations of heritable trait pairs

Overlooked genetic correlations between primary and secondary traits of the selection technique may complicate the selection because such traits generally produce either direct or indirect (either positive or negative) effects on the individual performance of the primary trait (de Almeida Silva et al., 2012; Lande & Arnold, 1983; Machado et al., 2017). Genetic correlation between two traits may happen due to unforeseen pleiotropism or gene binding imbalances exerted by one or both alleles and thus, such correlated traits pass down together through generations (Gresset et al., 2014; Machado et al., 2017). Consequently, high positive genetic correlation between traits of interest may shorten selection rounds in plant breeding programs, particularly when the environmental variance is small and also the opposite effect is possible with negatively correlated trait pairs (Nguyen & Sleper, 1983). Hence, the knowledge of genetic correlation is helpful when identifying beneficial drought-response trait associations and physiological selection criteria for plant improvement programs. In the current

experiment, the key plant traits that accounted for the highest proportion of variability in PCA, namely, WUE, SDW (including PDW and RGS), OP, and RDW_D were highly interrelated (r_A= >0.7) at $p < 0.05$. This suggests that selecting PRG for WUE should enhance the population with several other beneficial drought tolerance traits (i.e. dehydration tolerance through OA, deep rootedness, and post-cutting regrowth) in PRG improvement programs, if consistency of results is established in field trials. The literature also showed that regrowth rate, efficient plant WU, OP, and RDW_D traits act synergistically in several crops (Blum, 2017; Blum & Tuberosa, 2018; Ekanayake et al., 1985). However, the genetic background of such traits in PRG is first discussed here for the data from the current experiment in the light of current knowledge.

6.5.2.4 Correlated response to selection of major traits

Hill (2010) asserted that genetic correlations between traits substantially change over cycles of selection according to the consequence of gene frequency change, if pleiotropic loci are present in quantitative trait loci of the target traits. If selection intensities are unaffected from generation to generation, CR% specifies the relative effectiveness of indirect selection, that is proportional to the genetic covariance or correlation of secondary traits to the primary trait (Grafius, 1978; Hill, 2010). Despite high genetic correlations observed for key plant traits ($r_A \geq 0.7$, $p < 0.05$) in the current experiment, CR% estimates of trait pairs were generally lower than that of the ΔG_c estimate of each trait from the single-trait selection at 30% selection intensity (i.e. $CR_{SDW\text{-}RDWD}$ was 4.29% while ΔG_c of WUE, SDW, and RDW_D were 7.33%, 6.58%, and 9.92%, respectively; Table 6.5). This implies that direct selection of a given PRG population for WUE or each of its proxy trait (i.e. OA; as represented by OP in this chapter where, $r_{OP\text{-}OA}$ = 0.91 of Experiment 4 data when OA values were calculated using OP and RWC measurement data (Cyriac et al., 2018)) seems pragmatic compared to spending resources unnecessarily for several correlated traits one time (this argument is also applicable to PC1 and PC2 of PCAs 6.1 and 6.2 as well). However, CR estimates of RDW_D-OP and RGS-RDW_D trait pairs showed an increase from the ΔG_c estimate of at least one trait in the pair, exhibiting the importance of RDW_D (together with SMC_D data as justified elsewhere) as a beneficial selection trait for PRG drought tolerance

(Table 6.5). Moreover, the OP-RDW_D trait correlation estimate appeared to be as high as correlation estimates observed between OP and other measured traits ($r_A \geq 0.8$, $p < 0.05$), meaning that RDW and OP traits are promising surrogate traits for WUE. Nevertheless, for direct selection, WUE displayed high potential amongst all measured traits for PRG drought tolerance under tested conditions. Moreover, WUE1 displayed higher estimates of h^2_n and ΔG_c than those of WUE2 (i.e. h^2_n and ΔG_c estimates of WUE1 and WUE2 were 0.95 and 0.89 and 14.43% and 7.53%, respectively, and $r_{AWUE1\text{-}WUE2} = 0.6$; Table 6.6 and Figure 6.5), suggesting that PRG can effectually be selected for WUE before the onset of drought. However, future work is needed to screen a much larger PRG HS family population for the key traits simultaneously at well-watered and drought conditions in field settings, to recommend WUE measured on unstressed plants as a viable selection criteria to plant breeders. Yet, this research provides valuable clues to the breeder, if PRG is to be subject to selection for drought tolerance, as it is the first to analyze the quantitative genetic parameters of WUE and associated traits in PRG in a representative summer drought cycle in New Zealand.

6.5.3 Evaluating the possibility of pre-drought selection of PRG for WUE and associated traits

Information on plant WU under both stress and non-stress conditions have long been utilized as criteria in scheduling irrigation for pasture species in Australasia and South Africa (Heermann et al., 1990; Truter et al., 2016). However, a separate well-watered treatment was not included in the current experiment as it would have halved the number of plant genotypes that could be tested. Therefore, Experiment 4 focused on gathering trait information of the HS family population at two consecutive measurement phases; when plants were near FC in early regrowth following defoliation (M1) and when the same plants were at around 60% FC later in the same regrowth cycle (M2).

Literature shows that selecting PRG genotypes before the onset of drought enables screening and crossing selected plants for target traits within the same season and this may increase the efficiency of the breeding process and reduces costs of crop improvement programs (Condon & Richards, 1992; Rebetzke et al., 2008; Richards et

al., 2002). Richards et al. (2002) asserted that it is advisable to screen the wheat crop for target traits under non-stress conditions, particularly when the shoot biomass or the harvestable yield is considered as the main breeding objective under imposed drought stress. Similarly, Richards (1996) stated that some of the greatest successes in wheat breeding were achieved by breeding wheat accessions in environments where water is non-limiting. However, it is important to note that annual crops may be different from perennial plant species including PRG. However, for WUE, pre-drought selection may be beneficial (evidently shown the same in current results; Section 3.4) in temperate pasture species including PRG because shoot biomass or herbage yield is directly involved in the WUE calculation.

6.5.3.1 Pre-drought selection for PRG water relations traits

Despite the investigation of quantitative genetic information on carbon isotope discrimination (Condon et al., 2004; Farquhar & Richards, 1984; Rosielle & Hamblin, 1981), literature related to early selection for WUE is limited for any crop species and is almost absent for pasture species (Johnson & Bassett, 1991). According to the current results, WUE and LWP traits amongst other traits in the tested HS family population showed a potential for pre-drought selection or section of PRG genotypes during early growth near FC. This result was based on high r_A estimates of each trait observed between M1 and M2 and higher genetic variances, h_n^2, and ΔG_C estimates of trait data collected at M1 than those collected at M2 (Table 6.6 and Figure 6.4). As described in Chapter 6.4.2 above, WUE seemed to be amenable to selection prior to the onset of drought. Apart from that, previous studies have proposed LWP as a reliable selection criterion for drought tolerance in several crops at mild, moderate and, severe stress conditions (Bolaños et al., 1993; Jongdee et al., 2002; Matin et al., 1989; Quisenberry et al., 1985) including PRG (Thomas, 1987, 1990; Thomas & Evans, 1989; Thomas & Evans, 1991; Wang & Bughrara, 2008). However, its potential for selection when soil moisture is non-limiting has seldom been discussed. Therefore, for PRG breeding purposes, consistency and wider applicability of the current results needs to be established.

6.5.3.2 Pre-drought selection for gas exchange parameters

Gas exchange measures are considered to be major determinants of intrinsic and instantaneous WUE measurements (Condon, 2020; Gilbert et al., 2011). However, research evidence is still lacking to relate such information on proxy measures of WUE to actual WUE. Current results suggest that SC and Pn, when they are measured under drought, are unsuitable selection traits (based on the lower significance of their h_n^2 estimates and high CV; Table 6.3) for screening a PRG population for WUE and drought tolerance. This seems to be at variance with most published literature because moderate to high heritability estimates of stomatal characteristics including SC have been documented in the literature (Clarke, 1997; Faralli et al., 2019; Jones, 1979; Shahinnia et al., 2016). Also, SC has widely been used as a selection criterion when selecting crops for drought tolerance under stress conditions (Chaves et al., 2003; Flexas et al., 2002; Quarrie & Jones, 1979; Rebetzke et al., 2001; Shahidi et al., 2017; Shahinnia et al., 2016) or under progressively increasing drought conditions (Yan et al., 2017).

One reason for conflicting results in the current experiment may be the lack of repetition of measurements to reduce the error component or the data noise as they were only based on one single instantaneous measurement performed for each of two fully-expanded youngest leaves of two representative tillers (of many) of each genotype for the practical feasibility. Another reason may be the spread of measurements through the day and between days (i.e. measurements for 400×2×2 samples spanned 4 days and were carried out in time windows from 10.00 am to 12 noon and 2.00 pm to 4.00 pm each day) that is unavoidable when working with a large breeding population like the HS family population used in the current experiment. Rebetzke et al. (2003) found that even the h_n^2 estimates of SC and Pn in wheat varied widely (from 0.06 to 0.70) depending on the time of the day the gas exchange measurements are made. Moreover, it is a well-known fact that gas exchange measurements are highly influenced by both the explained and unexplained sources of variation (e.g. micro-environment) in different growing seasons (Section 2.2.4.4). Thus, phenotypic data collected even under similar soil drought treatments (i.e. 55–65% FC) in the series of glasshouse experiments of this

research may well have displayed an inconsistency in the gas exchange trait performance across experiments to the degree that was explained above and below.

In Experiment 1, 2, 3, and 4, phenotypic data from all measured traits including gas exchange parameters) and nine selected traits excluding Li-COR data to understand OP-WUE trait relationship better were compiled in two different PCAs ('Type1' and Type2', respectively, terms are used for easy identification of the two types of PCA hereafter) and then, correlations of PC scores of the first few PCs of both PCAs were compared in each experiment. For a better understanding for the gas exchange trait behaviour under imposed drought across experiments, it is important to compare Pn and SC data distribution patterns together with those of other trait data in selected PCAs. In Experiment 1, it was clear that the inclusion of gas exchange data in the 'Type1' PCA generally caused data redistribution among selected PCs and formed some modified PC structures and trait associations in each 'Type2' PCA. For example, Table 3.A2 (Chapter 3; Experiment 1) elucidates how the addition of new traits including gas exchange parameters modified the characteristics of the key trait association identified by PC3 of PCA3.1 (Type1; Table 3.1) in PCA 3.2 (Type2; Table 3.A1). With the addition of gas exchange data, the main observation was that PC3 of PCA3.1 in Experiment 1 ('Type1' PCA) that captured the key drought-response pattern was demoted to PC4 in PCA3.2 ('Type2') PCA with less data variation explained compared to the former. Conversely, when the HWUE and LWUE genotypes were selected based on PC3 of PCA3.1 in Experiment 1, a minor data variation explained by that PC became a major data feature in PCA3.2. When the gas exchange data (together with CF data) were included in PCA4.2 in Experiment 4, it was seen that 'WUE-OP' trait association of PCA4.1 was sub-divided in to 'WUE-gas exchange' and 'WUE-OP-RDW_D' trait associations (i.e. PC1 and PC3 of PCA4.2, respectively), with major differences in the PC coefficients of SMC_D, RDW_T, and RGS. However, the latter elucidated PRG drought tolerance and contributed to true WUE together with low RDW_T, high regrowth, and conserved soil moisture as described elsewhere. When gas exchange traits were excluded from PCA4.2, the PC3 separation became more important and was promoted to PC1 while the PC1 separation of that PCA was demoted and became PC3 in PCA4.1. This variable influence of major gas exchange data, when

analysed together with water relations trait data, implies that such instantaneous trait data are not always reliable for selecting PRG genotypes for actual WUE. However, PC scores of the first three PCs of PCAs 6.1 (Type1) and 6.2 (Type2) (Table 6.2) were significantly correlated (i.e. $r > 0.6$, $p < 0.05$; Table 6.A1), unlike the gas exchange trait performance observed in previous Experiments 1, 2, and 3.

SC has effectively been used to screen crops for drought tolerance under non-stress (Basnayake et al., 1995; de Almeida Silva et al., 2012; Fischer et al., 1998 ; Lu & Zhang, 1998). Nevertheless, the most appropriate measurement phase, if SC can be employed as one of the key selection criteria of plant drought tolerance, is still an unanswered question. In the current experiment, on a PRG HS family-mean basis, most quantitative genetic parameters estimated for SC and Pn were significant at M1 (i.e. SC1 and Pn1, respectively; $p < 0.05$, Table 6.6) and that was the complete opposite when data measured at M2 were considered ($p > 0.05$, Table 6.3). Also, SC1 showed a strong genetic correlation with both SDW1 ($r_A = 0.90$) and that was weaker but significant for SDW2 ($r_A = 0.12$) at $p < 0.05$. Moreover, the correlated response of SC-SDW trait pair at M1 was as high as the ΔG_c of each trait at M1. Apparently, SC will be a potential selection criterion for PRG drought tolerance, if the pre-drought selection is adopted. Stomatal conductance measurements taken before the onset of drought apparently exhibit high genetic variances, h_n^2, and ΔG_c estimates and significant direct and indirect trait associations made with SDW, WUE, and LWP traits at both M1 and M2, as observed in the current experiment under the conditions tested (Table 6.6). However, it is a must to eliminate experimental inaccuracies mentioned above in similar research in future in order to define the potential of SC for selecting PRG genotypes under non-stressed conditions.

6.6 Conclusions

Among 14 plant traits measured in 36 PRG HS families under both M2 and M1, phenotypic means of WUE and associated traits varied over a wide range. Results of ANOVA showed significant among- and within-HS family genetic variances confirming an adequate genetic diversity within the test population to facilitate an effective selection followed by a prospective population improvement for the key traits.

High h_n^2 estimates observed for the traits of interest further indicated the genetic potential of each trait for a direct and effective selection with high ΔG_c in successive generations. Estimated moderate to high ΔG_c values at an assumed 30% selection pressure for among family selection for WUE and also for SDW, RDW_D, OP, and RGS traits, from within the 36 HS families tested, indicated that selection for WUE would potentially be successful.

High estimates of r_A (typically above 0.7) between the key plant traits suggested the possibility of indirect selection or selecting an easily-measurable proxy trait for PRG WUE. However, estimated CR% values for those traits were generally lower than the ΔG_c estimates of each trait from the single-trait selection at an assumed 30% selection intensity, implying that the direct selection for WUE in a given PRG population is preferable if the logistical challenges can be overcome.

Statistically significant genetic variances and high h_n^2 and ΔG_c estimates observed for WUE when measured at M1, together with its high r_A estimates between M1 and M2 suggest that a PRG population can effectively be selected for WUE before the onset of drought. However, it should be noted that the quantitative genetic estimates from this study were generated under glasshouse conditions based on an evaluation for 36 HS families from a single population. Thus, it is advisable to conduct a similar experiment with a larger random sample of HS families from different breeding populations across multiple environments prior to recommending selection for WUE on un-droughted plants to PRG breeders. As the final step of the objectives set in a series of experiments mentioned above, results from this experiment showed that the major drought tolerance traits including SDW or RGS, WUE, RDW_D, and OP that were established in previous experiments are highly likely to be transferred to future generations with higher genetic gains. Thus, the current research provides valuable clues to breeders when selecting PRG for drought adaptation as, so far as the author is aware, this is the first study to evaluate key quantitative genetic parameters of WUE and associated traits of a PRG breeding germplasm, in a representative summer drought cycle in New Zealand.

Current results also suggest that future work is needed for a detailed investigation on gas exchange measures to determine the potential of using SC as a criterion in the selection of PRG for drought tolerance before drought stress occurs.

Chapter 7

General discussion

7.1 Introduction

The improvement of PRG offers direct opportunities to enhance pastoral sector performance. As stated in Chapter 1, PRG is New Zealand's primary forage grass species on which a NZD 20 billion export industry is based. In view of emerging climate change issues, efficient WU of PRG in summer moisture deficit is becoming important for the year-round productivity of pastoral agriculture despite the fact that WUE is historically a less-researched topic. Thus, this PhD research was set up, comprising four glasshouse pot experiments aimed at investigating 'WUE and drought tolerance in PRG'. Starting from the middle of 2017, a three-year study explored a test methodology aimed at evaluating PRG physiological trait responses under a soil moisture deficit challenge representative of field conditions faced by PRG plants in summer. Morpho-physiological and water relations traits related to a key drought tolerance trait response of PRG that could be used for pasture breeding purposes were studied and analyzed in the current research. Experiment 1 screened a population of three commercially available PRG cultivars, all carrying AR1 endophyte and with an industry track record of persistence for natural differences in morpho-physiological traits linked to WU under moderate drought (i.e. M2; 55–65%FC) (Chapter 3). Experiment 2 retested high and LWUE and HWUE genotypes from Experiment 1 under similar conditions in a second growing season, for consistency of the trait expression of elite plants across the two successive growing seasons (Chapter 4). Experiment 3 (inter-randomised with Experiment 2) evaluated the trait performance of the divergently selected LWUE and HWUE plants, in comparison with a plant breeder's elite plants from their breeding programme and with PRG germplasm from a naturally more arid Mediterranean origin (Chapter 5). Experiment 4 repeated the test methodology from Experiments 1 using a breeding population of 'HS families' from a germplasm archive. In Experiment 4, the average performance of seedlings from the same mother plant was compared across 36 different parent plants, to establish the quantitative genetic parameters including the heritability of the key traits, to inform plant breeders what

traits can feasibly be changed through selection (Chapter 6). The results from these four experiments provided a detailed understanding of the key drought tolerance trait response that lead to efficient WU in PRG under imposed drought and an insight into the relative importance of the genetic potential of each key trait to be transferred to succeeding generations. Additionally, representative PRG leaf samples from Experiment 4 were analyzed for carbon isotope discrimination ($\Delta^{13}C$) to address the research question 'Are proxy measures of agronomic WUE (i.e. instantaneous WUE and $\Delta^{13}C$-based intrinsic WUE) fully representative of the actual measurement of WUE in PRG? (Although these data are not presented in this thesis, the relevant paper that is to be submitted to a peer reviewed journal is in final draft stage of preparation at the time of writing).

Consistent data sets for the closely and functionally linked water relations traits from clonally identical PRG plants (carrying the same endophyte) grown under well-managed drought experiments are sparse in the literature. Also, information on heritability estimates of PRG WUE and associated morpho-physiological traits is almost absent from literature describing pasture cultivar selection programs. Therefore, the present data set appears to be almost unique. In this chapter, the background of the major findings of the four experiments in this PhD research project and also, their implications in related fields, and recommendations for follow-up research will be discussed.

7.2 Water-use efficiency and associated traits for PRG drought tolerance

Estimation of actual WU for pasture production is very important to farmers. Therefore, the knowledge of plant WU mechanisms, plant sensitivity to water stress, and strategies to achieve WUE have long been involved in irrigation scheduling for pasture species (Heermann et al., 1990; Truter et al., 2016). However, most scientists and plant breeders have often focused on the individual leaf-level WUE (i.e. intrinsic WUE) that corresponds to the instantaneous gas exchange measurements (Condon, 2020; Rebetzke et al., 2002) or carbon ($\Delta^{13}C$) or oxygen ($\Delta^{18}O$) isotope discrimination as a precise, heritable, and an easily measurable proxy for the actual WUE (Adiredjo et al., 2014; Ebdon & Kopp, 2004). Even so, the question 'Do these proxy measures truly capture

the WUE signal in terms of WU per unit of DM produced in a given plant?' still remains unanswered.

The current research centered on evaluating actual WUE in PRG tested under simulated drought conditions in a controlled environment. The major research objective was set based on the key results of a series of glasshouse experiments and a rainout shelter experiment conducted by PhD students at Massey University, New Zealand, in the past few years. In particular, the earlier research discovered that one prospect for future PRG research would be the selection of elite PRG genotypes displaying high WUE (Hussain, 2013) to realize drought tolerance of market-available PRG germplasm. Specifically, Hussain (2013) found that several genotypes of a commercial PRG cultivar (Grasslands Samson) displayed high WUE through maintaining high growth together with reduced depletion of SMC under water deficit in warm conditions. A follow-up pilot study conducted using 27 plants each of a "time series" of three commercial cultivars (i.e. Nui, Request, and Trojan) in the summer of 2014/2015 confirmed a wide range of WUE values for individual plants within cultivars (see 1A.1). This preliminary study also developed a practical methodology to provide a moisture deficit challenge to single PRG plants over a period of about a month and measure the water consumption and yield in a simulated summer water deficit. Accordingly, the current study was conducted to confirm previous findings within a context of PRG breeding perspective. For that reason, the initial selection in the current research (i.e. Experiment 1) had 50 or more PRG plants in each sub-group of the test plants (i.e. two clonal replicates of 220 genotypes making 440 plants from three cultivars namely Nui, Samson, and Trojan; Chapter 3) to establish reliable mean values for the traits of interest in the source cultivars (Sartie et al., 2009), and to screen as many genotypes as could be feasibly managed. With the tested 440 plants, this selection experiment also aimed at stimulating a drought cycle that is sufficiently representative of field conditions faced by pasture plants in summer in a controlled environment. This aim was further supported by the choice of 'Egmont black loam' soil with WHC > 65% (measured as MW/MS) that allowed sufficient time for a gradual intracellular physiological adjustment in test plants during a four-week soil moisture deficit from the first reduction in water supply.

It is important to have sufficient population diversity for the target trait to facilitate an effective selection, with high response to selection in each selection cycle (Blum, 2011; Chapman & Edmeades, 1999). There has been a reasonable number of experiments studied the phenotypic variability for the proxy measures of WUE (i.e. intrinsic WUE) in both forage and turf type PRG populations under imposed drought (Cui et al., 2015). However, published literature that discussed such information on actual WUE in studied species is sparse and that related to PRG cultivar selection programs is almost non-existent. Some authors suggest that this may be due to the practical difficulties of estimating a plant's WU precisely in field settings (Feldman et al.) or its doubtful physiological relationship with yield under drought conditions (Blum, 2005; Blum, 2009). However, as one of the key results of the current research, it was found that plant material included in Experiment 1 (i.e. a "time series" of three commercial PRG cultivars), and Experiment 3 (i.e. CBL and MMEL) had a large population diversity for WUE under the test conditions. This indicated that WUE may be a potential selection trait for PRG drought tolerance, if it is (1) an independent trait or synergistic component in a favourable drought-response trait association of elite PRG genotypes and (2) heritable, irrespective of its so-called limitations for high throughput measurements.

7.2.1 Eco-physiology of PRG water relations trait responses in response to drought

Selection of plants for drought tolerance must be pursued with due diligence to avoid possible trade-offs between physiological mechanisms that are synergistically or antagonistically linked to plant WU (Blum, 2005; Tuberosa, 2012). Blum (2009) argued that selecting plants for low WU (or WUE) will simultaneously decrease yield through a range of dehydration-tolerance strategies induced under moisture deficit conditions and often involving a reduction in growth processes. Blum (2009) also highlighted that effective use of water (EUW), which implies maximal soil moisture capture (apparently supported by OA) for transpiration, reduced non-stomatal transpiration or minimal water loss by soil evaporation, should be the focus in plant breeding programs, rather than WUE. In contrast to Blum's (2009) recommendation, PCAs that were compiled for the diverse WU and morpho-physiological trait data in each experiment in the current

research identified plant physiological processes comprising associations of multiple traits, some of which aligned with Blum's concept of effective use of water (e.g. PC1 of PCA 3.1) and some of which indicated WUE arising from high yield with low water consumption (e.g. PC3 of PCA 3.1) under imposed drought. In particular, an OP-WUE trait association since it comes in various associations with other traits (especially RDW_D and SMC_D) considered of interest for further research was identified as the major contributor to the drought tolerance property of a subset of PRG genotypes in all PRG germplasm lines evaluated in this research, which is also the subject of a lengthy discussion in this chapter. To facilitate that, different properties of the ecophysiological signature of water deficit response of PRG in the current results were synthesized and a model PRG plant including all desirable drought tolerance trait attributes for achieving high WUE was conceptualised (Figure 7.1) to highlight which traits are more relevant for this discussion and future research focus.

7.2.1.1 Morpho-physiological responses

Over many years of breeding work, pasture plants have generally been selected for the yield and phenotypic characteristics (i.e. visual scoring) or agronomic performance (Hatier et al., 2014; Lee et al., 2012). Nevertheless, yield is the most debated selection trait in terms of plant stress tolerance, particularly within the plant breeding framework (Blum, 2011). Reduced yield potential of pasture plants due to environmental stress conditions is primarily defined by a combined impact of reduced plant size, LER, leaf or tiller appearance, leaf area, and leaf area index similar to other plants (Barker & Caradus, 2001). These unfavorable plant responses are governed by complex plant physiological mechanisms that are induced for moderating leaf WU and plant water uptake under stress conditions (Abid et al., 2018; Blum, 2005; Chaves et al., 2003; Tuberosa, 2012). Therefore, most crop breeding programs have tended to focus on physiological selection criteria to define 'plant ideotype' in the target environmental stress (Blum, 2011; Richards, 2006). In particular, Annicchiarico et al. (2016) asserted that plant physiological traits that are related to the environmental stress tolerance are most likely to benefit future forage breeding programs, particularly under decreased and erratic rainfall events arising from climate change.

The most widespread and possibly effective plant drought tolerance strategy can be categorized as 'dehydration avoidance' that is identified by direct measurements of plant WU under imposed drought (Blum). Hence, the current research included measurements of physical traits related to soil moisture exploration by PRG roots, leaf WU measurements, and also measurement of physiological traits related to both plant water uptake and leaf WU. Results from Experiments 1 and 2 (Chapter 3 and 4), showed that the key drought-response pattern of evaluated PRG genotypes (i.e. WUE-OP trait association) was consistent across growing seasons. With additional parameters measured in Experiment 2, a specific drought tolerance trait response was identified where turgor maintenance by more negative OP was reinforced by HMWWSC accumulation in shoots together with a restrained pattern of deeper rootedness (i.e. presumably indicating efficient water extraction potential through deeper rootedness without proliferation of upper roots) and these traits led to high leaf elongation, tiller appearance, regrowth, and herbage yield and that is implied by high WUE of the elite germplasm under the

conditions tested. Results from Experiments 3 and 4 (Chapter 5 and 6) further elaborated that WUE, SDW, RGS, OP, SMC_D, and RDW_D are closely and functionally linked traits in the mentioned drought tolerance strategy. Also, it was found that plant-to-plant variation in RDW_T (or RSR) is likely to determine the rate of soil moisture drawdown in deeper soil layers and the degree of post-drought recovery of elite PRG plants in a prolonged drought. Accordingly, OP-WUE trait association functions in two ways: (1) WUE is achieved through improved plant hydration and high dry matter allocation to both shoots and roots with the involvement of high net assimilation at the expense of soil moisture (e.g. PC1 of PCA4.2; Chapter 4) and greater soil water extraction linked to more negative OP and deep rootedness as factors that keep plants growing longer in a drought; and (2) for reasons not fully established at this point in time, a reduction in water consumed per unit of shoot DM grown evidenced by trait associations similar to those in PC1 of PCA 4.2 (i.e. large plant size, and high tissue hydration), but reduced depletion of SMC_D together with a strong signal for more negative OP and without the involvement of gas exchange (e.g. PC3 of PCA4.2; Chapter 4). The fact that these alternate trait responses for high WUE are identified in different PCs in both Experiment 1 (PCA 3.1, PCs 1 and 3) and Experiment 2 (PCA 4.2 PCs 1 and 3), indicates that they are independent of each other as scores for different PCs in the same PCA are uncorrelated. The first approach is similar to Blum's interpretation of WUE as 'effective use of water' (i.e. low WU per unit of shoot tissue grown achieved by strategies that maintain active growth and avoid drought-induced yield constraint) or 'effective use of water' (as explained elsewhere). Blum (2009), points out that plants that manage to increase their water supply in order to keep growing longer into a drought will thereby allocate an increased proportion of WU for growth rather than leaf canopy maintenance (Blum, 2009). Within the same approach, maintenance of assimilation and growth under reduced plant WU in a drought may also include some unexplained plant physiological trait responses. For example, Flexas et al. (2016) asserted that maintaining high Pn under drought, while improving plant intrinsic WUE (i.e. lower plant evapotranspiration through partial stomatal closure) does not essentially require adjusted SC but it may also be achieved through either high mesophyll conductance and/or improved biochemical capacity (e.g. Rubisco properties) for CO_2 assimilation. However, those measurements were not recorded in this research. The second scenario exemplifies a

trait response that confers true WUE with which SMC_D is conserved but SDW is still high with or without the involvement of Pn. From a PRG breeding perspective, the latter trait association is imperative (Figure 7.1) and that was the reason why this trait response was the basis for the HWUE selection using PC3 scores of PCA 3.1 in Experiment 1 in the current research. For this second trait response scenario, mesophyll conductance may be a contributing factor to maintained or increased Pn at low SC.

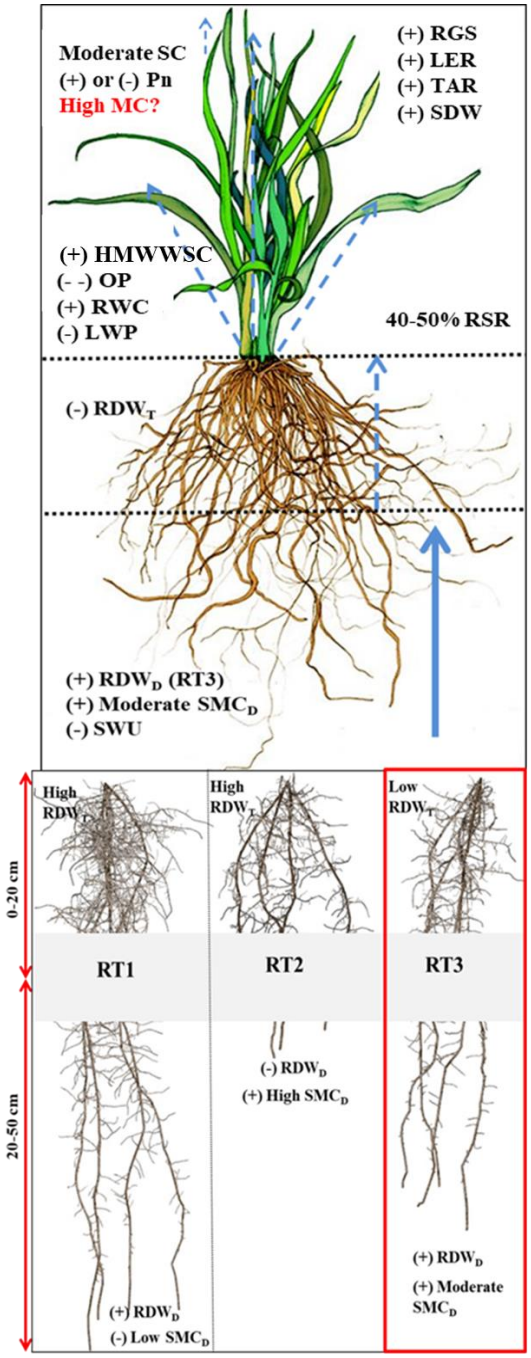

Figure 7.1 Identifying key traits of a model PRG plant for high WUE based on the ecophysiological signature of water deficit response in the current results. Abbreviations: SDW, Shoot dry weight (g/plant); SWU, Plant soil WU (g Water/pot); RT, Root type; RDW_T, Root dry weight at 0–20cm depth (g/plant); RDW_D, Root dry weight at 20–50cm depth (g/plant); RSR, Percentage total root: shoot ratio; SMC_D, Percentage gravimetric soil moisture content at 30–40 cm depth after post-cutting regrowth (%,w/w); LWP, Predawn Leaf water potential (MPa); OP, Osmotic potential (MPa); HMWWSC, High molecular weight water soluble carbohydrates (mg/g); RWC, Leaf relative water content(%); Pn, Photosynthesis (µmol/m^2/s); SC, Stomatal conductance (mmol/m^2/s); MC, Mesophyll CO_2 conductance. (Note: RT3 represents the ideal combination of RDW_D and RDW_T for a balanced RSR and efficient SWU).

The subtle but important differences in drought tolerance trait responses (1) and (2) both depend on a plant's ability to keep more negative OP under imposed drought. For reference, the magnitude of the PC coefficients of PC1 (Type 1) and PC2 (Type 2) in PCA6.1 for OP and simultaneous coefficients recorded for RDW_D, RDW_T, and RSR can be compared (i.e. for PC1, –0.226, 0.282, 0.289, and 0.105, respectively, and for PC2, –0.380, 0.301, –0.178, and –0.297, respectively; Table 6.2). Morgan (1984) asserted that increased RSR is a common drought adaptive response in plants due to weak OA, which allows plants to increase the water availability to the roots through decreased leaf WU and presumably also increase water capture. However, increased RSR can have many causes and in PC2 of PCA6.1, the indication was that plants displaying high WUE had decreased RSR, evidently through decreased RDW_T (which would reduce overall plant carbon demand) but with increased RDW_D (which would still provide for increased water capture). The key role of OA in response to soil drought, as identified by many authors, is to allow the plant a continuous reduction in LWP without a complete turgor loss, for enabling plant roots to maintain soil water uptake through a moisture gradient (Blum, 2017). While OA is generally measured in leaves, roots also adjust osmotically, which enhances preferential root growth and root water capture in deeper soil layers in a tactically targeted manner (Velázquez-Márquez et al., 2015). It was found that the ability of a plant to exhibit OA in the extension region of roots is higher (i.e. deep rootedness) than that of other plants at low LWP (Hsiao & Xu, 2000; Ober & Sharp, 2007). Also, cellular functioning of the lateral fibrous roots is generally restricted while longitudinal growth of roots including the taproot (in species such as alfalfa) progresses (i.e. most cell elongation and cell differentiation zones are located near the apex) under severe water deficit (Velázquez-Márquez et al., 2015). Similarly, WUE was found to be linked to root water uptake efficiency of some crops in previous research (Fan et al., 2008). These points of research evidence suggest that root turgor induced by more negative OP may well have contributed to favorable root adaptations of elite plants. Most experiments that assessed leaf water status by OA under conditions that ascribe a yield advantage do not include in-situ root or soil moisture observations taken at different soil depths, particularly in field settings. The current results suggest that the inclusion of in-situ root mass and soil moisture measurements at different rooting depths along with the records of herbage yield and plant water status may better inform selection of PRG plants for

drought tolerance, or at least is necessary to distinguish plants using drought-response patterns (1) and (2) above to achieve WUE.

7.2.1.2 Biochemical responses

When osmotic compounds measured in the current research are considered, proline accumulation seemed less important to OP, which was assumed to be correlated to OA of elite genotypes under conditions tested (Figure 4.7). However, (Blum, 2011) asserted that there is no known successful use of proline accumulation in breeding plants for drought resistance apart from some evidence documented for the over-expression of proline in studied drought-resistant plants in the literature. Therefore, LMW and HMW water soluble carbohydrate fractions were given more weight than proline in the discussion section of Chapter 4. In the current results, it was found that HMWWSC accumulation contributes to root and shoot growth dynamics in the absence of net assimilation under imposed drought, which is in turn an integral component in the OP-WUE trait response. In particular, elite genotypes exhibited high levels of HMW sugars and low levels of LMW sugars in growing leaves under test conditions (Figure 4.7). Previous research reported that preferential assimilate partitioning was encouraged by a signaling mechanism of different WSCs that were accumulated in studied crops under drought (Koch, 1996; Smeekens, 1998). For instance, (Blum, 2017) noted that photosynthetic products, mainly LMW sugars, are briefly accumulated in leaf cells first and then, transferred to root cells to induce OA in below-ground plant parts (Chimenti et al., 2006; Morgan, 1984). This may lead to a reduction in leaf LMW sugar levels in shoots or above ground plant parts while Pn is not affected as much under water deficit. Alternatively, (Sandrin et al., 2006) found that the metabolism of elongating leaf blades was primarily associated with the rate of accumulation of HMW sugars (mostly fructans) and that such accumulation led to high LER in two tested cool-season grass species. Accordingly, it can be suggested that PRG shoots and roots respond differently to sugar accumulation and remobilization, particularly for adjusting OP in a mutually-supportive manner under imposed drought. Similarly, several authors found that sugar concentration in plant tissues has an important physiological role under drought where sugar responsive genes have control over resource distribution among tissues and organs (Koch, 1996; Smeekens, 1998). It has also been found that sugar depletion in shoots, due to assimilate transportation to extending roots, upregulates

genes for photosynthesis and/ or remobilization or export of assimilates to growing tissues (Koch, 1996; Smeekens, 1998). This drought response may well have contributed to high LER, TAR, and regrowth rate observed in elite genotypes, which exhibited more negative leaf OP together with low LMW and high HMW sugar accumulation in the current research.

7.2.1.3 Gas exchange physiology: stomatal and non-stomatal limitations

As the soil moisture dropped from FC to 60%FC or 45%FC, SC and Pn of both low and high WU PRG genotypes showed a drastic decline in all experiments. Apparently, turgor maintenance by OA was unable to support net assimilation of elite genotypes under tested conditions as suggested elsewhere. Serraj & Sinclair (2002) asserted that the potential role of OP in drought tolerance is well-identified in plants grown under severe drought stress conditions at which gas exchange is already restricted by non-hydraulic signals (i.e. ABA hormonal signals generated at the initial stages of drought as noted in Chapter 2). Hence, several authors asserted that SC can be a sensible selection criterion for drought tolerance, if plants are selected under irrigated conditions (Faralli et al.). However, the current research did not corroborate this speculation. Possible reasons behind this contradiction were explained in Section 6.5.3.2 where unavoidable inaccuracies in measurements and unexplained variations in the micro environment that the test plants were grown. It may also be that there are multiple mechanisms for reduced SC, and it is logical to think that more negative OP might reduce evaporation of water from cell surfaces into intercellular spaces and in this way reduce leaf water loss at the same stomatal aperture (Blum, 2017).

Stomatal conductance has long been debated with respect to its potential as a selection trait for plant stress tolerance (Chapter 2). In particular, early research asserted that there has been a limited success achieved in incorporating desirable stomatal characteristics into improved cool-season grass cultivars due to the inconsistent behavior of stomata under field conditions (Johnson & Asay, 1993). Nevertheless, recent research suggested that selecting crops for stomatal conductance may provide the plant breeder with potential benefits in terms of its ability to regulate water loss and net assimilation, that may result in improved WUE under drought (Condon, 2020; Flexas et al., 2016). However, this theoretical

approach raises questions such as; (i) to what extent this leaf-water saving strategy contributes to an efficient plant WU in a progressively increasing drought? and (ii) can the leaf water-saving benefit due to stomatal closure outweigh the possible assimilation loss or vice versa?

As noted elsewhere, intrinsic WUE is generally conferred by SC and photosynthetic capacity of a given plant. However, several authors have asserted that up-scaling from intrinsic WUE to whole plant-level or agronomic WUE is absolutely complex (Condon, 2020). The supplementary study of Experiment 4, that was conducted to explore how reasonable is to use of Δ^{13}C-based or gas exchange-based intrinsic WUE data as a surrogate for directly-measured WUE, revealed that intrinsic WUE can only explain a 10% proportion of variation in WUE under the conditions tested (Weerarathne et al., unpublished data). Also, the literature explains the uncertainty in interpreting intrinsic WUE that is calculated based on Δ^{13}C or gas exchange measurements, with an unclear involvement of MC (Condon, 2020). However, the current research did not record MC to define this so-called unsolved mystery of low SC and apparently high MC trait response. Even so, results of the supplementary experiment mentioned above demonstrated that there may be an indirect or weak trait association between intrinsic WUE and WUE, which could arise through a genetic linkage between OP and SC (Weerarathne et al., unpublished data). This was further supported by the high r_A estimates observed between SC, OP, LWP, and RWC traits and intrinsic WUE in the same study. The same result also suggested that, if PRG genotypes are selected for this combination of traits, the degree of representation of intrinsic WUE in actual WUE may be increased from 10% to 50%. However, these findings warrant further research at both the physiological and genetic or molecular level, for confirmation of the results in a wider context and for a comparison with current results due to the unavailability of documented evidence to support the presented results.

Apart from the adaptations in stomatal behavior, gas exchange physiology of the elite HWUE genotypes seemed to be acclimatized to the progressively increasing drought through non-stomatal adaptations. For example, CF parameters of elite genotypes were tested at 45–55% FC in Experiment 2 to reveal another facet of the relative ability of the elite germplasm to tolerate consequences of an intense drought. The focus of this analysis was to use CF data for a detailed physiological

interpretation of water relations data (Chapter 4). As expected, these results corroborated the key results where HWUE genotypes exhibited higher Fv/Fm, F'v/F'm, φPSII, qP, and ETR values and lower NPQ value than that of the LWUE selection in Experiment 2. It was also found that these parameters can be stand-alone criteria for determining the leaf-water status and gas exchange physiology of drought-exposed PRGs plant in an intense drought event (refer to PC1 of PCA4.2; Table 4.1). However, it was clear that CF parameters would not provide substantive information on soil moisture status or efficient water uptake, if they were not combined with SMC_D measurements when selecting a PRG germplasm for drought tolerance (i.e. PC3 of PCA4.2 that explicated the key drought-response pattern or OP-WUE-SMC_D trait association did not capture CF information). The practical importance of each mentioned CF parameter for plant drought tolerance is well-understood, as set out in Chapter 2. As a whole, CF parameters amongst many other non-stomatal drought indicators (i.e. lipid peroxidation and oxidative stress) have long been identified as reliable and an independent set of selection criteria for screening plant germplasms (Li et al., 2006) including temperate grasses (Cielniak et al., 2006) for drought tolerance at extreme stress levels. In addition, enzyme-antioxidants and malondialdehyde concentration could also be good indicators for quantifying the degree of damage to plant cells and tissues at intense drought (Abid et al., 2018; He, 2016; Manavalan et al., 2009). Such measurements may provide an in depth understanding to the cell-level drought-response patterns of stressed pasture plants.

Considering recent summer drought scenarios that have occurred in New Zealand, information on non-stomatal limitations seems less important for PRG breeders. This is basically because autumn rains usually alleviate summer drought impacts on pasture growth before drought intensifies to extreme levels. In more severe drought events there may be brown-off of the sward where new leaf production may appear to cease and existing leaves senesce, with recovery after drought coming from resumption of leaf production and tiller bud release, though the author is not aware of any published detailed description of post-drought recovery processes. However, such trait measurements may be important for pasture selection programs targeting extreme climate events in future, if pasture persistence, year-round production, and sustained regrowth are prioritized over the capacity to maintain productivity for

longer as a drought develops. Also, screening elite genotypes that can tolerate extreme moisture deficits may benefit the farmer in coming years because climate predictions have indicated that major pasture production regions in New Zealand will experience 5–10% more drought occurrences by the year 2050 than have occurred in the recent past (Johnston, 2013).

7.2.2 Quantitative genetics of WUE and associated traits

The literature highlights two major challenges of physiological selection approaches in breeding forage species for drought tolerance; (1) collection of precise and consistent physiological data in field trials (Annicchiarico et al., 2016) and (2) selection of elite parents through the exploitation of the maximum genetic variability of a given plant population for beneficial physiological traits under imposed drought (Annicchiarico et al., 2016; Bertan et al., 2007). In both cases, the data quality may be considerably influenced by environmental effects that mask the true genotypic value (i.e. G × E effects) (Acquaah). Thus, (Blum, 2011) suggested that 'pre-breeding', that involves the testing and validation of the key physiological traits down to the details of the measurement protocols under a managed stress environment, may be a potential preparatory stage for embarking on a physiological selection approach. However, documented evidence for such research is almost absent from the literature describing pasture cultivar selection programs. Therefore, the main focus of the current research was to take initiatives for bridging these research gaps.

Blum (2009) argued that genotypic variation in WUE is primarily driven by variations in growing conditions affecting the degree to which growth occurs while water is being transpired for canopy maintenance and cooling processes (unlikely to be heritable or a source of production gain if selected for). It was further explained that the mentioned plant drought response does not occur through a physiologically-driven variation in plant production or assimilation per given amount of WU that is shown in this study to be heritable. Blum (2009) strongly developed the argument that dehydration avoidance is primarily characterized by the maintenance of high plant hydration to support post-drought survival which is the complete opposite of a high yield potential genotype. A trait association between high WUE and low SDW was identified in PC2 of PCAs 3.1 (Table 3.3) and 4.1 (Table 4.1), and this

illustrates the effect that Blum (2009) was concerned about when selecting plants for high WUE as discussed elsewhere in previous chapters. This explains the fact that plants rather maintain small size with less WU than increasing DM production with efficient WU, as a water conservation strategy to defend against drought. However, as noted above, another independent trait association that was clearly seen in this study and was not recognized by Blum (2009) was first explained in PC3 of PCA3.1 and then, PC1 of PCAs 3.3 (Chapter 3), 4.1 (Chapter 4), 5.1 (Chapter 5), and 6.1 (Chapter 6). This particular trait association (as explained elsewhere) was consistent with data for Samson cultivar by Hussain (2013) where there was genuinely a physiological mechanism allowing water saving concurrently with continued growth (i.e. true WUE). As noted above, the current work indicates that more negative OP can be expressed either by enhanced water capture and depletion of SMC_D for more effective use of water as advocated by Blum (2009), or by enhanced leaf DM production per unit of WU, and conservation of SMC_D. Furthermore, Experiment 4 of the present study provides quantitative genetic data indicating to breeders that the target traits are likely to be passed to succeeding generations with high genetic gains under the conditions tested.

Chapman & Edmeades (1999) and (Acquaah, 2012) described the importance of using populations which have a high population mean and sufficient genetic variability for the primary trait (i.e. yield) and as well as for the secondary traits (i.e. selected drought-tolerant traits including WUE) that are relevant to the expected plant ideotype in the target environment. In the current research, population mean for SDW or herbage yield was generally high because the test PRG germplasm lines used in all four experiments were deliberately chosen from commercial cultivars and advanced breeding lines. Also, results from the series of experiments in this research showed within-population variation for WUE and different morpho-physiological traits measured in different PRG populations. Moreover, phenotypic variation observed for the WUE trait and associated traits in HWUE genotypes in Experiment 1 were consistent across two growing seasons, indicating that those traits may be reliable selection criteria for PRG drought tolerance. Hence, the current results did not support claims in the literature that it would be inadvisable to use WUE or indicators of dehydration avoidance such as OP, as selection traits for plant drought tolerance (Blum, 2005; Blum, 2009). Thus,

it was considered that WUE as the primary trait and associated morpho-physiological traits (i.e. OP, RWC, SMC_D, RDW_D, and RGS) as the secondary traits in all experiments irrespective of the fact that WUE and OP traits performed two uncorrelated trait responses under imposed drought as indicated in the results of PCAs in each Chapter. Accordingly, Chapter 6 set forth how the quantitative genetic information of the key traits is used to realize the relative degree of genetic control over the phenotypic performance of each trait.

For cross-pollinating forage grasses, the first step in planning and conducting breeding programs is to obtain information as to the size and nature of the genetic variation and the genetic relationship between the chosen traits (Casler & Brummer). Such information is expressed in terms of statistical parameters for genetic variance components, broad-sense and/ or narrow-sense heritabilities, genetic correlations, G × E effects, response to selection, and predicted and realized genetic gains of the target traits (Casler & Brummer, 2008; Wilkins & Humphreys, 2003). For PRG, progenies of poly-crossed individuals or clones are extensively used to extract quantitative genetic information of target traits (Casler & Brummer, 2008). Experiment 4 explored major quantitative genetic parameters of the key traits using a breeding population of HS families from a germplasm archive under a well-managed drought environment (Chapter 6). Results displayed high genetic variances (i.e. within and between half-sib families) and high h_n^2 estimates (i.e. ranged from moderate to high; 55–96%) and as well as, low CV (highlighting low experimental error) values for WUE, SDW, OP, regrowth, and RDW_D traits under imposed drought. Thus, current results emphasize the potential of the WUE trait and introduced morpho-physiological traits for improvement through selection targeting drought tolerance. This is reinforced when noting moderate to high ΔG_c estimates observed for WUE, SDW, OP, regrowth, and RDW_D traits under imposed drought in Experiment 4 (Chapter 6). However, new selection techniques may not be considered or immediately applied to the main stream selection protocol of a breeding program by pasture breeders.

Blum (2011) asserted that novel findings should be tested for more than one season. Nevertheless, plant breeders are generally conservative in their general approach of obtaining 'realized genetic gain' of introduced traits that is to be estimated through several selection cycles in different environments for more

convincing results than that with the 'predicted genetic gain' estimated in one season (Blum, 2011; Rutkoski, 2019). Alternatively, there should be sufficient evidence for a yield gain or loss of the germplasm of interest compared to that of a benchmark cultivar or an elite germplasm, when it is screened using newly introduced selection traits (preferably with similar irrigation input and microclimate to that is faced by plants in field conditions in summer) (Blum, 2011). In the current research, this was somewhat achieved by evaluating the HWUE selection for water relations traits in comparison to that in two elite PRG germplasm lines from a commercial pasture breeder's breeding program (i.e. CBL and MMEL that were tested separately in field trails by a commercial seed company for yield and several other properties of commercial value) (Chapter 5). These three germplasm lines were inter-randomized in the same glasshouse space for testing under a simulated summer drought condition in Experiment 3, as in Experiment 1. Results showed that the performance of multiple traits in the key drought response (i.e. SDW-WUE-OP-RDW_D-RDW_T-SMC_D-RGS) was very similar across the elite plants of the three germplasm lines compared (Chapter 6). Results also suggested that there can be a significant improvement in the typical field scoring system, if it is combined with morpho-physiological traits related to PRG WU in the selection strategy adopted in glasshouse experiments and proven heritable in the current research.

7.3 Further research

The discussion arising from results of Experiment 2 (Chapter 4) included two major points of speculation (1) mesophyll conductance may provide a substantive contribution towards the maintenance of net assimilation and growth of elite PRG plants that adjust SC (i.e. partial stomatal closure) as a leaf-water and soil-water conservation strategy (i.e. PC3 of PCA4.2; Table 4.1) and (2) osmotic adjustment in roots (apparently contributed by accumulation of LMWWSC and HMWWSC while interconnected to leaf osmotic adjustment) may well have triggered preferential DM partitioning between top and deeper roots of elite plants for enhanced soil water access and balanced RSR (i.e. low RDW_T and high RDW_D). Either of these effects or both acting together might have led to higher SMC_D and RSR ranging from 40% to 50% of in elite plants under imposed drought (see Figure 7.1 above). In the literature, both the postulated effects above are mentioned as possible physiological

trait responses, as noted in Section 4.3.1.1. For example, Section 7.2.1.2 above notes that preferential assimilate partitioning between above-ground and below-ground plant parts may be mediated by a signalling mechanism of different water soluble carbohydrates that are generally accumulated in both shoots and roots of drought-exposed plants. However, relevant measurements to elucidate these possible effects were not made in the current research. Thus, it would be worth exploring the physiological trait responses indicated in points (1) and (2) above, in order to ascertain if they play a role in the drought tolerance trait association identified in the series of experiments in this research.

Several authors argued that trait measurements should be performed under non-stressed conditions to improve heritability and the selection efficiency for yield and target drought-tolerant traits (Richards, 1996; Richards et al., 2001; Richards et al., 2002). Current research explored genetic information of the expression of key traits in both situations throughout the growth and development of test plants (i.e. FC and progressively increasing drought intensity at early and later canopy regrowth stages). The results showed that WUE and LWP traits amongst other traits have the potential for selection at early growth stages near FC, based on high genetic correlation estimates of both traits observed between FC and 60%FC conditions and also, higher genetic variances, and ΔG_c estimates observed for each trait at FC than that at 60%FC under managed environment (Figure 6.4; Chapter 6). This novel finding deserves attention and further investigation by forage grass breeders as early selection enables screening and crossing of selected plants for target traits within the same season and in this way increases the efficiency of the breeding process and resource use (Condon & Richards, 1992; Rebetzke et al., 2008; Richards et al., 2002). Nevertheless, Blum (2011) asserted that that heritability estimates obtained under stress environments can be acceptable, if the selection environment is well-managed (i.e. controlled environment that is free from light, shade or soil moisture gradients and weed, pest or disease occurrences) and the source population contains sufficient genetic variability for yield and secondary traits that are relevant to the target environment and breeder's target ideotype.

The current research established significant quantitative genetic estimates for the key water relations traits at 60% FC under a controlled environmental condition (i.e. simulated one single New Zealand summer drought cycle) based on an evaluation

for 36 HS families from a single population. For practical implications and wider applications, there are two limitations in the current results as highlighted in the literature: (1) newly identified traits should not be adopted as selection criteria based on genotype performance in just one environment because of possible G×E interactions (Blum, 2011) and (2) quantitative genetic analyses that are performed on only a few genotypes or on genotypes derived from a limited number of parents may result in low estimation accuracy (Fè et al., 2015). As noted in Chapter 2, drought is a complex environmental stress. Apart from soil moisture deficit, drought impacts on plants may also depend on a number of factors such as soil properties, plant characteristics, and microenvironmental factors including temperature and humidity or water vapour deficit that were not completely emulated in the current research. Thus, considerable G×E effects might be displayed by selected genotypes in field settings where these environmental variations are unavoidable. Furthermore, drought damage can be aggravated by the magnitude and frequency of drought occurrences. Considering these facts, it is clear that there is a need for future research that evaluates similar information in a wider application (i.e. AWFS in a larger breeding population/s under multiple soil moisture challenge events) across multiple field environments prior to recommending heritable selection criteria introduced in this research for forage plant breeding purposes. Further research is also needed to establish realized genetic gain in the case of a multi-trait selection such as the trait association identified as the key drought-response pattern of PRG elite plants comprised multiple trait associations that synergistically govern PRG WUE (i.e. concurrent selection for WUE, OP, RDW_D, SMC_D, and RGS traits).

7.4 Conclusions

The current research identified a novel trait association in PRG plants conferring high WUE and drought tolerance. Under the conditions tested, more negative leaf OP associated with increased HMWWSC accumulation is linked to efficient soil moisture uptake and leaf WU, and that improved summer WUE may underlie yield improvements in modern cultivars. Further experiments confirmed that the trait association identified was consistently expressed in clones of selected elite plants on retesting in the following growing season. A further experiment to obtain quantitative genetic information, indicated that the traits of interest, considered singly, are highly heritable and therefore relevant for consideration as selection

criteria in PRG improvement programs. However, all glasshouse pot experiments conducted in the current research consisted of manageable numbers of PRG plants (<450) in order to keep the experimental error to a minimum and testing and validation of results was for one simulated summer drought cycle for practical feasibility. Thus, it is suggested that expanding the current research to retest the genetic background of the key traits including WUE in a much larger PRG population, in multiple drought cycles in the field, would be advisable before making recommendations to PRG breeders based on the current results. Convincing results from such field trials would largely benefit the dairy industry in New Zealand. New Zealand dairy farmers are the main users of new cultivars and regularly resow their pastures on a cycle of about every ten years in many cases. The milk production curve is similar across years, irrespective of summer rainfall, indicating that supplementary feeds are purchased when grass does not grow because of water deficit. This means any gain in WUE will reduce farmer feed costs in dry summers in proportion to the herbage yield gain, under any given level of water deficit, of PRG cultivars with enhanced WUE. Therefore, this pre-breeding work lays a strong foundation to establish a selection approach in commercial PRG breeding programs that will help to mitigate the adverse consequences for pasture productivity of projected future climate scenarios where summers are on average warmer and drier than at present.

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Appendices

1A.1 Abstract of a short communication that was prepared by the writer for submission to a peer-reviewed journal based on data collected from the 'proof of concept' study by visiting Brazilian researchers Carnivalli and Garcia in 2014.

Discerning component traits contributing to specific water use in perennial ryegrass

Visna Weerarathne[1*], Larissa Garcia[2], Roberta Carnevalli[3], Bingzhe Fu[4], Cory Matthew[1]

[1]School of Agriculture and Environment, PN433 Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand

[2] Luiz de Queiroz College of Agriculture, University of São Paulo, Av. Pádua Dias, 235 - Agronomia, Piracicaba - SP, 13418-900, Brazil

[3]Brazilian Agricultural Research Corporation, Ministry of Agriculture, Livestock, and Food Supply, Brazil 78550-570

[4]School of Agriculture, Ningxia University, 489 He Lan Shan West Rd, Yinchuan, China 7500213

ABSTRACT

Productivity of perennial ryegrass (*Lolium perenne* L.) is constrained by summer water deficit. We hypothesized that selection for desirable water relations traits might partially mitigate this constraint. Genotypes of a 1970s, and two recent ryegrass cultivars were grown from transplanted tillers in 55 cm tall pots for three months and exposed to moisture deficit for four weeks, then 66 plants were assessed for shoot and root dry weight, leaf elongation rate (LER), specific water use (SWU, reciprocal of water-use efficiency), leaf osmotic potential (OP), OP change (OP_{dif}), relative water content (RWC), and gravimetric soil moisture. Trait associations were evaluated by correlation and principal component (PC) analyses. Among the 66 genotypes, SWU ranged from 935±19 to 1754±74 g H_2O g^{-1} DM. In PC analysis, PC1 indicated that greater LER and SDW and lower SWU from greater soil water extraction were the common trait responses of the large majority of genotypes tested under imposed drought. PC2 indicated water saving by decreased LER and SDW, with increased root mass, but with high SWU indicative of summer dormancy. PC3 associated more negative OP with improved SWU and RWC. This experiment demonstrates not only genotypic diversity for SWU, but also diversity in trait associations determining SWU, which should be considered when investigating SWU, both in ecology and agronomy research.

KEYWORDS**:** Genotypic diversity, leaf osmotic potential, perennial ryegrass, plant water relations, principal component analysis, soil moisture extraction, specific water use, water-use efficiency

3A.1 Information related to the standard curve that was prepared for each chamber of the Wescor C–52 by measuring a series of NaCl solutions with different molarities (0.2, 0.4, 0.6, 0.8, 1.0 mol) as described in the manual for OP measurements.

(A) Water potentials of NaCl solutions at temperatures between 0−40°C and the calibration equations for each C-52 chamber

TABLE I

WATER POTENTIALS OF NaCl SOLUTIONS AT TEMPERATURES BETWEEN 0-40°C

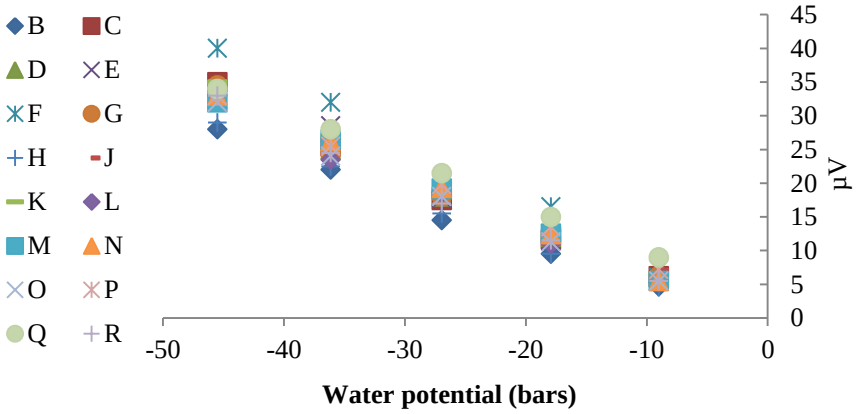
(Lang, 1967)

Temperature / Molality	0°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C	40°C
0.05	-2.14	-2.18	-2.22	-2.26	-2.30	-2.34	-2.38	-2.42	-2.45
0.1	-4.23	-4.31	-4.39	-4.47	-4.54	-4.62	-4.70	-4.77	-4.85
0.2	-8.36	-8.52	-8.68	-8.84	-9.00	-9.15	-9.30	-9.46	-9.61
0.3	-12.47	-12.72	-12.97	-13.21	-13.44	-13.68	-13.91	-14.15	-14.37
0.4	-16.58	-16.93	-17.27	-17.59	-17.91	-18.23	-18.55	-18.86	-19.17
0.5	-20.70	-21.15	-21.58	-22.00	-22.41	-22.81	-23.22	-23.62	-24.02
0.6	-24.84	-25.39	-25.93	-26.44	-26.94	-27.44	-27.94	-28.43	-28.91
0.7	-29.01	-29.67	-30.30	-30.91	-31.51	-32.10	-32.70	-33.28	-33.85
0.8	-33.20	-33.98	-34.72	-35.43	-36.12	-36.82	-37.51	-38.18	-38.85
0.9	-37.43	-38.32	-39.17	-39.98	-40.79	-41.58	-42.27	-43.14	-43.90
1.0	-41.69	-42.70	-43.66	-44.59	-45.50	-46.40	-47.29	-48.15	-49.01
1.1	-45.99	-47.13	-48.20	-49.24	-50.26	-51.27	-52.26	-53.22	-54.18
1.2	-50.32	-51.60	-52.78	-53.94	-55.07	-56.20	-57.30	-58.35	-59.41
1.3	-54.70	-56.11	-57.42	-58.69	-59.94	-61.19	-62.39	-63.54	-64.71
1.4	-59.12	-60.68	-62.10	-63.50	-64.87	-66.23	-67.54	-68.80	-70.06
1.5	-63.59	-65.29	-66.84	-68.37	-69.86	-71.34	-72.76	-74.11	-75.48
1.6	-68.11	-69.96	-71.63	-73.30	-74.91	-76.52	-78.05	-79.50	-81.07
1.7	-72.60	-74.60	-76.40	-78.20	-80.00	-81.70	-83.30	-84.90	-86.50
1.8	-77.30	-79.40	-81.30	-83.30	-85.20	-87.00	-88.80	-90.40	-92.10
1.9	-81.90	-84.30	-86.30	-88.40	-90.40	-92.40	-94.30	-96.00	-97.80
2.0	-86.70	-89.20	-91.30	-93.60	-95.70	-97.80	-99.80	-101.60	-103.50

WATER POTENTIAL (BARS)

(B) This table from C-52 instruction/ service manual shows the relationship between different NaCl molality and water potential at different temperatures. In our experiment, the temperature was around 20−22°C, therefore the corresponding water potential at 20°C was regarded as providing the X values in the following graph

which shows the standard curve for each chamber. The calibration equation for each chamber is displayed below.



Chamber No.	Calibration Equation
B	y = −1.5331x − 2.9633
C	y = −1.2582x − 2.9872
D	y = −1.402x − 0.7077
E	y = −1.249x − 3.0392
F	y = −1.0768x − 2.3283
G	y = −1.295x − 2.6177
H	y = −1.4708x − 3.2382
J	y = −1.3193x − 0.3915
K	y = −1.2609x − 2.0026
L	y = −1.3313x −2.7582
M	y = −1.3585x − 1.0923
N	y = −1.3257x − 1.5349
O	y = −1.3846x − 1.9497
P	y = −1.4321x + 1.2612
Q	y = −1.4474x + 4.0249
R	y = −1.3313x − 2.7313

Table 3.A1 Principal component analysis of the same data used in PCA3.1 with the addition of data for gas exchange traits (PCA3.2) to make a data matrix containing plant morphology, water relations, and gas exchange data for 16 trait measurements of 186 perennial ryegrass genotypes from three cultivars; Nui, Samson and, Trojan.

	PC 1	**PC 2**	**PC 3**	**PC 4**	**PC5**	**PC6**
Eigenvalue	3.99	2.36	1.87	1.30	1.14	0.94
% variation explained	25.0	14.8	11.7	8.1	7.1	5.9
Cumulative % variance	25.0	39.7	51.5	59.6	66.7	72.5
SDW	0.287	0.184	0.309	0.332	–	–0.369
WUE	–0.249	–0.214	–0.403	–0.298	0.104	0.301
LWP	–0.135	0.211	0.246	–	0.224	–0.227
OP	–0.165	–0.280	0.450	–0.218	–	–
RWC	–	0.100	–0.531	0.287	–	–0.162
SMC_D	–0.245	–0.327	–0.114	0.276	0.242	–
RDW_D	0.209	0.420	–0.105	0.304	–	0.256
RDW_T	0.295	0.372	0.111	–0.109	–	0.185
RGS	–	–	–0.145	0.543	0.358	0.114
Pn	–0.308	0.196	0.182	0.179	–	0.229
SC	–0.366	0.254	–	–	–	0.142
Ci	0.351	–0.324	–	–	0.331	0.186
Ci/Ca	0.361	–0.331	–	–	0.287	0.173
ET	–0.216	–	0.265	0.220	0.404	0.279
VPD_L	–0.281	0.136	–	–	0.438	–
T_L	–0.102	0.144	–0.166	–0.321	0.432	–0.595

Trait abbreviations: SDW, Shoot dry weight; WUE, Water-use efficiency (g Water use/g DM); LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC, gravimetric soil moisture content at 30–40 cm depth; RDW_T, Root dry weight at 4–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth; RGS, Post–cutting regrowth score; Pn, Photosynthesis; Ci, Intercellular CO_2 concentration; Ci/Ca, The ratio between Ci and atmospheric CO_2; SC, Stomatal conductance; ET, Evapotranspiration; T_L, Leaf temperature; VPD_L; Leaf vapor pressure deficit. (Note: Negative coefficients for WUE indicate less WU/g DM).

Table 3.A2 Cross-correlations of PC scores of the first five PCs between PCA3.1 and PCA3.2 performed for the nine water relations trait data of the 220 means of 440 genotypes from Trojan, Samson, and Nui cultivars (two clonal replicates of 90, 80, and 50 genotypes, respectively) at M2 (55−65% FC) in Experiment 1.

	PCA3.2				
PCA3.1	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**
PC1	0.710[R]	0.695	0.077	−0.059	0.037
	0.000[P]	0.000	0.297	0.427	0.622
PC2	0.161	−0.118	0.947	0.158	−0.037
	0.030	0.113	0.000	0.032	0.621
PC3	0.053	−0.164	0.087	−0.872	−0.041
	0.478	0.027	0.240	0.000	0.582
PC4	0.025	−0.039	0.010	0.225	−0.390
	0.742	0.597	0.896	0.002	0.000
PC5	0.079	0.014	−0.024	0.044	0.384
	0.290	0.856	0.749	0.558	0.000

(Note: [R] = Pearson correlation coefficient; [P] = p-value)

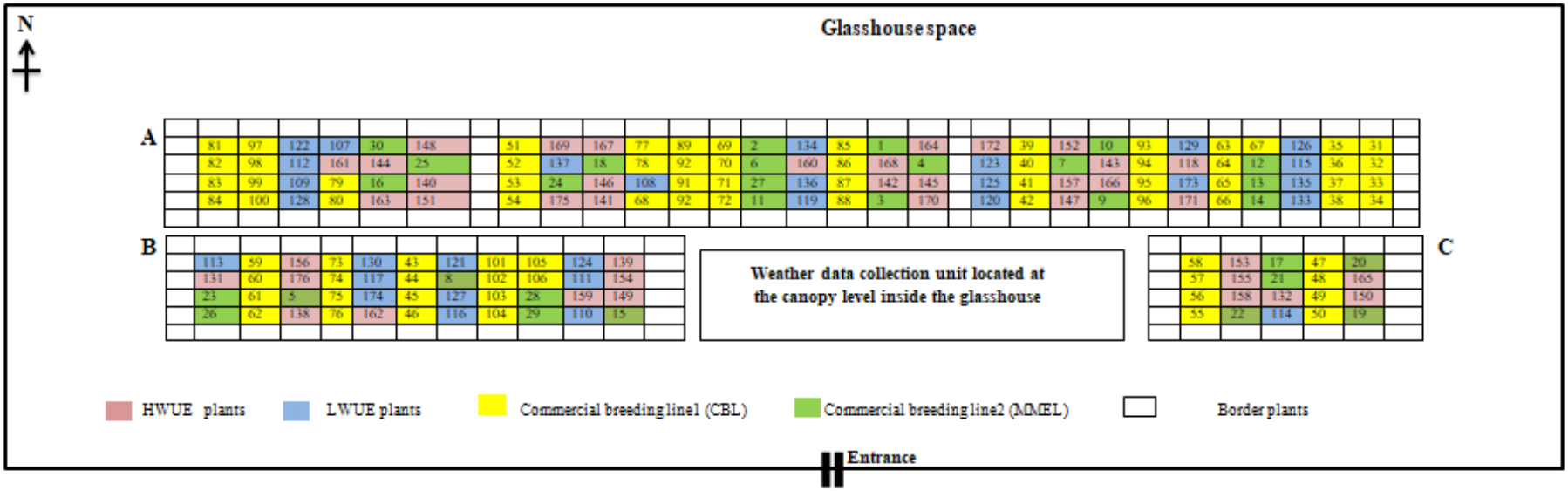


Figure 4.A1 Experiment layout of Experiments 2 and 3: Inter-randomisation of potted ryegrass genotypes from four different germplasms (as represented by different colour cells with pot numbers in the figure and identified in the figure legend) used in a the same glasshouse space for both experiments (Note: HWUE and LWUE denote low water use and high water use selections, respectively, from Experiment 1)

The data collected at the mini weather station include; Glasshouse temperature, °C; Incident light, W/m^2; PAR, W/m^2; RH, %.

Table 4.A1 Principal component co-efficients of the first five PCs of PCA3.3 that was performed for the nine water relations traits, as in PCA3.1 and PCA4.1. The data are for the 35 selected HWUE and LWUE genotypes were measured at M2 (55–65% FC) in Experiment 2.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Eigen value	5.48	1.39	0.63	0.49	0.43
%variation explained	60.9	15.4	7.0	5.5	4.8
%Cumulative variance	60.9	76.4	83.4	88.9	93.7
SDW	0.395	0.200	0.640	−0.152	−0.154
WUE	−0.379	0.430	−0.163	−	0.381
LWP	−0.348	0.289	−0.167	−0.248	−0.480
OP	−0.393	0.148	−0.260	−	−
RWC	0.151	−0.359	0.373	0.182	−0.136
SMC_D	0.317	−0.665	−0.232	−0.296	−0.170
RDW_D	0.394	−0.240	0.126	−0.298	−
RDW_T	−0.257	0.558	0.350	−	0.224
RGS	0.284	−	−	−0.510	−0.118

Trait abbreviations: SDW, Shoot dry weight; WUE, Water–use efficiency; LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; $SMC_{D,}$ Gravimetric soil moisture content at 40–50 cm soil depth; RDW_D, RDW at 20–50 cm depth; RDW_T, Root dry weight at 10–20 cm depth; RGS, Post-cutting regrowth score.

Table 4.A2 Cross-correlations of PC scores of the first five PCs between PCA 4.1 (Experiment 2) and PCA 3.3 (Experiment 1) performed for the nine water relations traits of the 35 selected genotypes at M2 (55−65% FC) in both experiments.

	PCA4.1				
	PC1	**PC2**	**PC3**	**PC4**	**PC5**
PCA3.3					
PC1	0.880[R]	0.111	0.122	−0.053	−0.169
	0.000[P]	0.527	0.485	0.762	0.333
PC2	0.070	0.781	0.081	−0.326	0.279
	0.691	0.009	0.644	0.05	0.104
PC3	0.173	−0.366	0.473	−0.266	−0.056
	0.321	0.031	0.025	0.123	0.751
PC4	−0.146	0.137	−0.061	0.565	0.135
	0.403	0.433	0.727	0.010	0.141
PC5	−0.017	−0.521	−0.289	0.184	0.708
	0.922	0.011	0.093	0.291	0.006

(Note: [R] = Pearson correlation coefficient; [P] = p-value)

Table 4.A3 Pearson correlation co-efficients of measured traits of selected 35 perennial ryegrass genotypes at M2 (55−65% FC) in Experiment 2

	WUE	SDW	TAR	LER	RDW_T	RDW_D	SMC_T	SMC_D	OP	LWP	RWC	RGS	Proline	LMW WSC	HMW WSC	Pn
SDW	–0.664[R]															
	0.000[P]															
TAR	–0.455	0.371														
	0.006	0.028														
LER	–0.542	0.350	0.837													
	0.001	0.039	0.000													
RDW_T	0.782	0.715	0.523	0.541												
	0.000	0.000	0.001	0.001												
RDW_D	–0.883	0.556	0.310	0.433	0.665											
	0.000	0.001	0.070	0.009	0.000											
SMC_T	0.081	–0.478	0.126	0.067	–0.422	–0.560										
	0.590	0.004	0.471	0.703	0.012	0.000										
SMC_D	–0.642	0.199	0.447	0.478	0.066	0.140	0.324									
	0.009	0.051	0.007	0.004	0.706	0.022	0.057									
OP	0.925	–0.549	–0.448	–0.559	0.789	–0.842	0.544	–0.340								
	0.000	0.001	0.007	0.000	0.000	0.000	0.101	0.030								
LWP	0.747	–0.412	–0.538	–0.582	–0.544	–0.647	0.437	–0.267	0.738							
	0.000	0.014	0.001	0.000	0.001	0.000	0.009	0.088	0.000							
RWC	–0.200	0.603	0.597	0.669	0.668	0.608	–0.345	0.238	–0.702	–0.643						
	0.150	0.000	0.000	0.000	0.000	0.000	0.043	0.081	0.000	0.000						
RGS	–0.914	0.718	0.412	0.458	0.765	0.890	–0.585	0.220	–0.875	–0.653	0.755					
	0.000	0.000	0.014	0.006	0.000	0.000	0.000	0.044	0.000	0.000	0.000					
Proline	0.395	–0.242	–0.471	–0.602	–0.240	–0.330	–0.227	–0.343	0.330	0.301	–0.462	–0.315				
	0.019	0.161	0.004	0.000	0.164	0.053	0.190	0.044	0.053	0.079	0.005	0.066				
LMWWSC	0.254	–0.139	–0.169	–0.035	0.190	0.284	–0.256	0.076	–0.246	0.023	–0.006	0.247	0.177			
	0.142	0.427	0.331	0.840	0.275	0.098	0.138	0.666	0.154	0.894	0.974	0.152	0.310			
HMWWSC	–0.893	0.576	0.555	0.646	0.816	0.823	–0.433	0.182	–0.927	–0.770	0.705	0.855	–0.382	–0.390		
	0.000	0.000	0.001	0.000	0.060	0.000	0.009	0.148	0.000	0.000	0.000	0.000	0.024	0.025		
Pn	–0.849	0.609	0.291	0.400	0.622	0.965	–0.590	0.154	–0.757	–0.589	0.595	0.882	–0.333	–0.279	0.744	
	0.000	0.000	0.089	0.014	0.000	0.000	0.000	0.152	0.000	0.000	0.000	0.000	0.050	0.054	0.000	
SC	–0.809	0.558	0.323	0.426	0.586	0.959	–0.528	0.187	–0.737	–0.593	0.582	0.855	–0.337	–0.236	0.733	0.989
	0.000	0.001	0.058	0.011	0.000	0.000	0.001	0.182	0.000	0.000	0.000	0.000	0.048	0.071	0.000	0.000

. (Note: [R] = Pearson correlation coefficient; [P] = p-value; Please see the next page for trait abbreviations).

Trait abbreviations: SDW, Shoot dry weight; TN, Tiller number per plant; TAR, Tiller appearance rate; LER, Leaf elongation rate; WUE, Water-use efficiency or g WU/g DM; LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC_T, Gravimetric soil moisture content at 10–20 cm depth; SMC_D, Gravimetric soil moisture content at 40–50 cm depth; RDW_T, Root dry weight at 0–20 cm soil depth; RDW_D, Root dry weight at 20–50 cm soil depth; RGS, Post-cutting regrowth score; LMWWSC and HMWWC, Low and high molecular weight water soluble carbohydrates; Pn, Photosynthesis; SC, Stomatal conductance.

Table 4.A4 Pearson correlation co-efficients estimated for the trait data of selected 35 perennial ryegrass genotypes between Experiment 1 and 2 that were measured at M2 (55−65% FC) in both experiments.

	SDW1	WUE1	LWP1	OP1	RWC1	SMC_D1	RDW_T1	RDW_D1	RGS1
SDW2	0.878[R]	-0.806	-0.649	-0.815	0.262	-0.599	0.554	0.825	0.546
	0.000[P]	0.000	0.000	0.000	0.128	0.000	0.001	0.000	0.001
WUE2	-0.838	0.756	0.665	0.877	-0.403	0.505	-0.359	-0.770	-0.415
	0.000	0.000	0.000	0.000	0.160	0.002	0.034	0.000	0.013
LWP2	-0.767	0.713	0.498	0.762	-0.345	0.453	-0.399	-0.671	-0.446
	0.000	0.000	0.002	0.000	0.042	0.006	0.018	0.000	0.007
OP2	-0.876	0.806	0.752	0.910	-0.496	0.451	-0.259	-0.794	-0.402
	0.000	0.000	0.000	0.000	0.002	0.007	0.133	0.000	0.017
RWC2	0.702	-686	-0.573	-0.765	0.311	-0.539	0.386	0.657	0.423
	0.000	0.000	0.000	0.000	0.069	0.001	0.022	0.000	0.011
SMC_D2	-0.312	0.297	0.021	0.232	-0.306	0.446	-0.136	-0.204	-0.242
	0.068	0.083	0.907	0.180	0.074	0.052	0.434	0.239	0.162
RDW_T2	0.712	-0.664	0.620	-0.786	0.429	-0.472	0.302	0.660	0.357
	0.000	0.000	0.000	0.000	0.010	0.004	0.078	0.000	0.035
RDW_D2	0.894	-0.768	-0.587	-0.749	0.275	-0.522	0.517	0.811	0.488
	0.000	0.000	0.000	0.000	0.110	0.001	0.001	0.000	0.003
RGS2	0.812	-0.763	-0.640	-0.843	0.390	-0.552	0.410	0.778	0.451
	0.000	0.000	0.000	0.000	0.021	0.001	0.015	0.000	0.007

(Note: [R] = Pearson correlation coefficient; [P] = p-value; Please see the next page for trait abbreviations)

Trait abbreviations: SDW, Shoot dry weight; WUE, Water-use efficiency or g WU/g DM; LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; SMC_D, Gravimetric soil moisture content at 40–50 cm depth; RDW_T, Root dry weight at 0–20 cm soil depth; RDW_D, Root dry weight at 20–50 cm soil depth; RGS, Post-cutting regrowth score.

Table 5.A1 Untransformed trait means and their standard deviation (SD) values of 38 genotypes of the commercial breeding line (CBL), a two-criterion selection of the best four CBL plants considering field score (as the prioritized feature) and glasshouse WUE (CBL-elite) namely, P19, P17, P21, and P37, the best four CBL plants considering high glasshouse WUE together with the highest possible field-score (CBL-HWUE) namely, P19, P17, P32, and P9, 14 plants of the Mediterranean cross (MMEL), and 20 genotypes of the high water-use efficiency selection (HWUE) from Experiment 1 and 2 as measured at M2 (55–65% FC).

Trait measurement	**CBL**	SD	**CBL-elite**	SD	**CBL-HWUE**	**SD**	**MMEL**	SD	**HWUE**	SD
SDW	5.60	0.13	6.29	0.44	6.21	0.11	2.23	0.21	6.47	0.18
WUE	516.1	32.7	527.8	185.8	326.1	77.5	563.8	53.9	555.8	45.08
OP	–2.68	0.05	–2.70	0.24	–2.9	0.12	–2.67	0.08	–2.77	0.06
LWP	–1.04	0.008	–1.03	0.01	–1.07	0.03	–1.05	0.001	–0.95	0.01
RWC	69.0	0.85	69.19	4.87	72.9	1.54	66.1	1.40	68.7	1.75
RSR	52.7	3.7	50.2	3.28	45.3	5.10	187.9	2.67	56.1	2.86
RDW_T	2.91	0.19	2.41	0.20	2.54	0.69	3.38	0.30	2.69	0.25
RDW_D	0.89	0.01	0.97	0.36	1.45	0.33	0.81	0.14	0.95	0.12
SMC_T	27.9	1.21	31.3	4.46	35.6	4.1	19.9	0.57	22.7	0.52
SMC_D	29.6	1.2	32.9	5.17	36.9	4.5	28.5	1.98	29.3	1.65
RGS	3.21	0.15	4.0	0.41	4.0	0.41	1.43	0.24	4.45	0.20
Pn	4.19	0.22	4.4	0.26	4.5	0.55	2.12	0.09	4.98	0.06
SC	0.15	0.005	0.16	0.01	0.15	0.01	0.12	0.005	0.14	0.002

(Please see the next page for trait abbreviations)

Trait abbreviations: SDW, Shoot dry weight; WUE, Water-use efficiency (g WU/g DM); OP, Leaf osmotic potential; LWP, Predawn leaf water potential; RWC, Leaf relative water content; RSR, Total root: total shoot ratio; RDW_T, Root dry weight at 0–20 cm depth; RDW_D, Root dry weight at 20–50 cm depth; SMC_T, Gravimetric soil moisture content at 10–20 cm depth; SMC_D, Gravimetric soil moisture content at 30–40 cm depth; RGS, Post-cutting regrowth score; Pn, Photosynthesis; SC, Stomatal conductance.

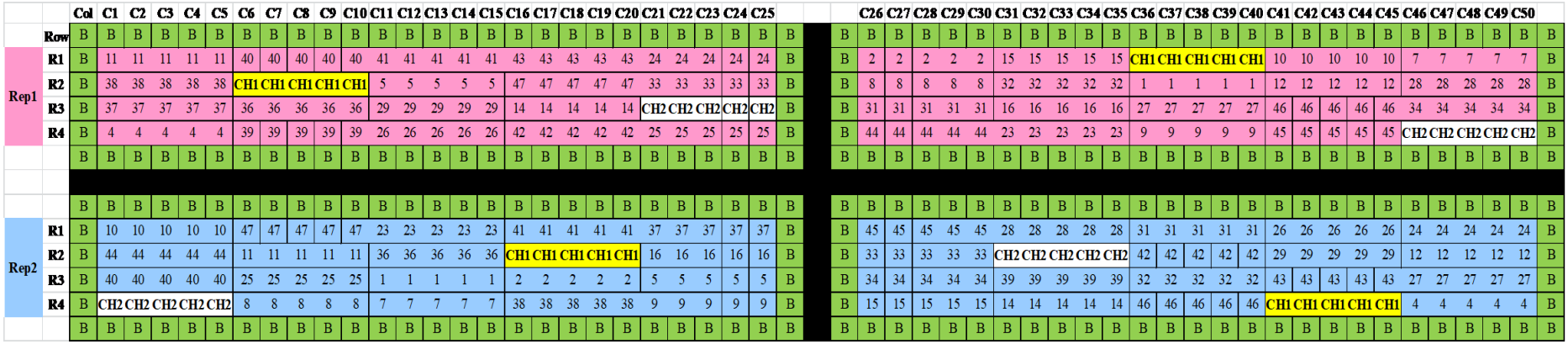


Figure 6.A1 The row (R)-column (C) experimental design incorporating two clonal replicates of 36 perennial ryegrass HS families from a breeding population as test plants, and four clonal replicates of two HS families as check plants (CH) to statistically test for spatial effects on plant growth. Each HS family was represented by five randomly selected genotypes. The border plants are indicated as 'B'. Numbers in black are HSF genotype identifiers.

Table 6.A2 Cross-correlations between the PC scores for the first five PCs of PCA6.1 and PCA6.2 performed for the fifteen and nine water relations traits, respectively, of the HS family population measured at M2 (Note: 180 genotype traits means of two clonal replicates of 360 test plants excluding check plants were compiled in both PCAs).

	PCA6.1				
	PC1	**PC2**	**PC3**	**PC4**	**PC5**
PCA6.2					
PC1	-0.983[R]	-0.052	-0.091	0.078	-0.064
	0.000[P]	0.497	0.231	0.306	0.403
PC2	-0.056	0.888	0.086	0.053	0.148
	0.463	0.000	0.258	0.081	0.100
PC3	0.057	0.053	-0.606	**-0.331**	**0.381**
	0.455	0.487	0.051	**0.002**	**0.001**
PC4	-0.129	**0.202**	**0.217**	-0.787	0.065
	0.089	**0.008**	**0.004**	0.045	0.395
PC5	0.019	0.046	-0.099	**0.126**	-0.210
	0.800	0.545	0.194	**0.098**	0.005

(Note: [R] = Pearson correlation coefficient; [P] = p-value)

Table 6.A2 Pearson correlation co–efficients of measured traits of 180 genotype means of two clonal replicates of 360 test plants (excluding check plants) of the HS family population, measured at M2 (Please see the next page for trait abbreviations and footnotes).

	WUE	SDW	RDW_{Tot}	PDW	RSR	RDW_T	SMC_T	RDW_D	SMC_D	OP	LWP	RWC	RGS	Pn
SDW	–0.866[R]													
	0.000[P]													
RDW_{Tot}	0.303	0.217												
	0.000	0.003												
PDW	0.732	0.702	0.669											
	0.000	0.000	0.000											
RSR	0.204	–0.250	0.771	0.100										
	0.006	0.001	0.000	0.182										
RDW_T	0.253	0.145	0.958	0.594	0.767									
	0.001	0.052	0.000	0.000	0.000									
SMC_T	–0.464	–0.367	–0.572	–0.623	0.221	–0.506								
	0.000	0.000	0.000	0.000	0.003	0.000								
RDW_D	–0.316	0.307	0.764	0.618	0.525	0.547	0.532							
	0.000	0.000	0.000	0.000	0.000	0.000	0.000							
SMC_D	0.652	0.686	–0.683	–0.709	–0.308	0.584	0.744	0.679						
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000						
OP	0.329	–0.205	0.288	–0.332	0.081	0.255	–0.299	–0.265	–0.828					
	0.000	0.007	0.000	0.000	0.289	0.001	0.000	0.000	0.000					
LWP	0.315	–0.291	0.602	–0.584	0.366	0.500	–0.597	–0.631	–0.733	0.265				
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
RWC	–0.254	0.168	–0.216	0.234	–0.115	–0.178	0.231	0.231	0.191	0.776	0.159			
	0.001	0.024	0.004	0.002	0.125	0.017	0.002	0.002	0.010	0.000	0.033			
RGS	–0.404	0.350	–0.649	0.629	–0.378	–0.536	0.629	0.689	0.748	0.337	0.827	0.239		
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001		
Pn	–0.189	0.142	0.104	0.140	0.061	0.069	0.179	0.148	0.113	0.512	0.112	0.812	0.159	
	0.011	0.057	0.166	0.061	0.413	0.359	0.016	0.048	0.131	0.000	0.133	0.000	0.033	
SC	–0.106	0.087	–0.013	0.042	–0.023	–0.022	–0.096	0.013	–0.114	–0.254	–0.018	0.557	0.038	0.687
	0.156	0.248	0.866	0.579	0.761	0.770	0.200	0.867	0.057	0.001	0.812	0.000	0.615	0.000

(Note: [R] = Pearson correlation coefficient; [P] = p-value)

Trait abbreviations: SDW, Shoot dry weight; WUE, Water-use efficiency or g WU/g DM; RDW_{Tot}, Total root dry weight; PDW, Total plant dry weight; RSR: Total root to total shoot ratio; SMC_T, Gravimetric soil moisture content at 10–20 cm depth; SMC_D, Gravimetric soil moisture content at 40–50 cm depth; RDW_T, Root dry weight at 0–20 cm soil depth; RDW_D, Root dry weight at 20–50 cm soil depth; LWP, Predawn leaf water potential; OP, Leaf osmotic potential; RWC, Leaf relative water content; RGS, Post–cutting regrowth score; Pn, Photosynthesis; SC, Stomatal conductance (Note: Phenotypic correlations of SMC_D with measured traits are important as it is a key perennial ryegrass drought tolerance but a non-plant trait with no genetic correlations computed with other traits).

Personal development activities linked to this research

- Preliminary results were presented at the SAE symposium, SAE, Massey University, New Zealand (2017).

- A poster titled 'Drought resistance in perennial ryegrass involves morphological plasticity-osmotic adjustment interplay' was presented at the Combio2018 conference, ICC, Sydney, Australia (24^{th} – 26^{th} September 2018).
  For reference: DOI: 10.13140/RG.2.2.33492.32640

- Experiment 1 results were presented at the NZGA conference, Twizel, New Zealand (2018).
  Reference to the conference paper: DOI: 10.33584/jnzg.2018.80.332

- Had an opportunity to have a PhD exchange visit for isotope ratio mass spectroscopy analysis of samples from Experiment 4, at the Technical University of Munich, Munich, Germany, from November 2019 to April 2020. This collaborative segment of research was funded by the DAAD (German Academic Exchange Service) Short-term Research Grants Scholarship 2019/20. The research proposal was first presented at the Grassland chair, School of Life Sciences, Technical University of Munich
  For reference: DOI: 10.13140/RG.2.2.31814.60481
  For MS PowerPoint slides: DOI: 10.13140/RG.2.2.23416.16643

- The abstract submitted to the Joint XXIV International Grassland Congress XI International Rangeland Congress that was scheduled to be held from 25^{th}-30^{th} October 2020, Nairobi, Kenya, was accepted last year. The full paper submission and the conference were postponed to the end of this year due to Covid-19 consequences so, we withdrew the paper.